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Effects of interaction between arbuscular mycorrhizal fungi, rhizobacteria, soil phosphorus and plant cytokinin content on tobacco growth

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Abstract

The rhizosphere is an important zone for microbial activity and diversity, where several beneficial associations between roots and microorganism occur, and is fundamental in terms of defining terrestrial food production. When growing in a complex and heterogeneous medium such as soil, roots develop in a structured but flexible manner to optimize their functions. Endogenous factor, such as plant physiology and genetics, as well as abiotic and biotic exogenous factors are important determinants. In the present study I hypothesized that the effects of soil microorganisms on root morphology are associated with plant growth and differ depending on endogenous phytohormone levels and soil nutrient availability. A full factorial experiment was set up in the greenhouse, with presence or absence of *Glomus intraradices* or *Pseudomonas fluorescens*, with two phosphorus amendments, and with the tobacco wild type (*Nicotiana tabacum*) or the *35S:CKX2* transgenic line with reduced cytokinin content. The effects of microorganisms on plant biomass changed from negative to neutral and positive depending on plant type, phosphorus amendment and microorganism presence, and are partially explained by alteration on root morphology. Mycorrhization was enhanced in the *35S:CKX2* mutant or in presence of *P. fluorescens*.

Key words: *Nicotiana tabacum*, *Glomus intraradices*, *Pseudomonas fluorescens*, root morphology, cytokinin, phosphorus.

Resumo

A rizosfera é uma zona importante em termos de actividade e diversidade microbiana, onde ocorrem associações benéficas entre plantas e microorganismos, e é fundamental na produção agrícola. Ao proliferarem num meio complexo e heterogéneo como o solo, as raízes desenvolvem-se de forma estruturada, mas flexível, por forma a optimizar suas funções. Factores endógenos, como fisiologia e genética da planta, bem como factores exógenos abióticos e bióticos são elementos determinantes. Neste estudo proponho testar a hipótese de que os efeitos dos microorganismos do solo sobre o crescimento da planta estão associados a efeitos sobre a morfologia radicular e dependentes dos níveis endógenos de fitohormonas e disponibilidade de nutrientes no solo. Uma experiência factorial foi instalada na estufa, com a presença ou ausência de *Glomus intraradices* ou *Pseudomonas fluorescens*, com dois regimes de fertilização de fósforo, e com a linhagem selvagem de tabaco (*Nicotiana tabacum*) ou a transgénica *35S:CKX2* com teor de citoquinina reduzido. Os efeitos dos microorganismos sobre a biomassa alternaram entre o negativo, neutro ou positivo, dependendo da linhagem, do regime de fertilização e a presença de microorganismos, e explicam-se parcialmente com alterações na morfologia da raiz. A micorrização aumentou na linhagem *35S:CKX2* ou em presença de *P. fluorescens*.

Palavras chave: *Nicotiana tabacum*, *Glomus intraradices*, *Pseudomonas fluorescens*, morfologia radicular, citoquinina, fósforo.

Resumo alargado

A rizosfera é considerado um dos lugares mais fascinantes em termos de actividade e diversidade microbiana e é uma zona importante e determinante para a qualidade e quantidade dos recursos alimentares terrestres. A "Revolução Verde" aumentou drasticamente a produção alimentar nos últimos 40 anos, mas o seu aumento foi acompanhado por um aumento no uso de agroquímicos trazendo diversos problemas ambientais. O estudo da rizosfera tem permitido a identificação de exemplos com grandes impactos agronómicos explicáveis em termos de interacções entre as raízes e os microorganismos do solo, evidenciando potenciais alternativas ao uso de agroquímicos.

As rizobactérias promotoras do crescimento das plantas beneficiam as plantas e promover o seu crescimento através de vários meios de acção. Os fungos micorrízicos arbusculares, um dos grupos de microorganismos do solo mais difundidos, que formam simbioses funcionalmente importante com espécies de muitas famílias de plantas terrestres, podem também promover a aptidão e o crescimento das plantas. As raízes são uma chave essencial nesta interacção micróbio-planta. As principais funções das raízes são a ancoragem da planta ao solo e a absorção de água e nutrientes. Ao crescerem num meio complexo e heterogéneo como o solo, as raízes desenvolvem-se de forma estruturada, mas flexível, para optimizar suas funções. Factores endógenos, como a fisiologia e a genética da planta, bem como factores abióticos e bióticos exógenos são determinantes importantes para a morfologia radicular.

No presente estudo, pretendo testar as seguintes hipóteses: 1.1) os efeitos de *Glomus intraradices* ou *Pseudomonas fluorescens* sobre o crescimento da planta de tabaco estão associados à alteração da morfologia da raiz, e 1.2) dependem tanto da disponibilidade de fósforo no solo como do teor endógeno de citoquinina na planta; 2.1) a micorrização aumenta na linhagem de tabaco com teor reduzido de citoquinina; e 2.2) uma rizobactéria originalmente isolada de uma rizosfera não micorriza pode igualmente promover a micorrização.

Uma experiência factorial foi instalada na estufa, com presença ou ausência de *G. intraradices* ou *P. fluorescens*, com dois regimes de fertilização de fósforo, e com a linhagem selvagem de tabaco (*Nicotiana tabacum* L. cv. Samsun NN) ou a linhagem transgénica *35S:CKX2* com teor reduzido de citoquinina. As plantas foram cultivadas durante dois meses, após os quais determinou-se a biomassa da planta, morfologia radicular, e perfil químico da parte aérea.

Nos controlos sem inóculo de microorganismos, a fertilização reduzida de fósforos diminui a biomassa da planta e aumentou o rácio raiz:parte aérea em ambas as linhagens, mas não promoveu qualquer alteração na morfologia radicular na linhagem selvagem. O rácio raiz:parte aérea não evidencia nenhum papel significativo na absorção de azoto. A morfologia radicular da linhagem *35S:CKX2* teve maior sensibilidade à fertilização, e as alterações na morfologia estão associadas a diferentes teores de azoto na parte aérea.

Na linhagem selvagem sob baixa fertilização de fósforo, *G. intraradices* reduziu a biomassa da planta, associado a uma redução do diâmetro radicular e aumento do comprimento específico radicular, no que parece resultar de uma relação competitiva entre planta e fungo. *P. fluorescens* também reduziu a biomassa da planta, associado a uma redução do comprimento total da raiz. Embora não tenha sido possível no presente estudo determinar a colonização radicular de *P. fluorescens*, e relacionar a sua densidade com a função inibitória, uma imagem é possível projectar entre esta função e o comprimento total da raiz ao longo dos diferentes tratamentos. Nos tratamentos em que o comprimento total de raízes é reduzido, a biomassa da planta diminui em presença de *P. fluorescens*. Quando o comprimento radicular não foi afectado ou tende aumentar, a biomassa da planta ou não é afectada ou tende a aumentar. Ainda sob fertilização de fósforo reduzida, a biomassa da linhagem selvagem não foi afectada quando co-inoculada pelos dois microorganismos. No entanto, a maioria dos parâmetros morfológicos radiculares foram reforçados, mas sem alterações no teor de azoto na parte aérea, sugerindo que a presença de ambos os microrganismos levou a planta a desenvolver o seu sistema radicular para sustentar a mesma absorção de azoto.

Sob fertilização de fósforo elevada, a biomassa da linhagem selvagem não foi substancialmente afectada pelas inoculações microbianas, nem tanto a sua morfologia radicular ou o teor de azoto na parte aérea. Estes resultados sugerem que os efeitos de ambos os microorganismos na biomassa da planta dependem da disponibilidade de fósforo no solo e estão associados a mudanças morfologicas radiculares.

Na linhagem *35S:CKX2* sob baixa fertilização de fósforo, *G. intraradices* não teve efeitos na biomassa, nem no comprimento, superfície ou volume total da raiz. No entanto, o diâmetro médio radicular aumentou e, consequentemente, o comprimento específico da raiz reduziu. O teor de azoto na parte aérea parece beneficiar do aumento do diâmetro médio radicular. *P. fluorescens* incrementou ligeiramente a biomassa, bem como o comprimento, superfície ou volume total da raiz, e tanto o

diâmetro médio radicular como o rácio raiz:parte aérea foram claramente incrementados. Tal como observado na linhagem selvagem sob baixa fertilização de fósforo, a biomassa de *35S:CKX2* no mesmo regime de fertilização não foi afectada pela co-inoculação. No entanto, este ultimo mostrou uma tendente redução nos parâmetros morfológicos radiculares, contrastando com o observado no tipo selvagem, sugerindo que a citoquinina teve um papel relevante na interação planta-micróbio e no aumento destes parâmetros morfológicos.

A biomassa de *35S:CKX2* sob elevada fertilização de fósforo foi reduzida por todas as inoculações, e o teor de azoto na parte aérea parece ter tido um papel determinante no crescimento da planta. Estes resultados rejeitam parcialmente a hipótese 1.1. Ou seja, apesar dos efeitos dos microorganismos na morfologia radicular estarem fortemente associados ao crescimento da planta, isto não é sempre verificável, e outros factores como a aquisição de nutrientes podem ter um papel igualmente determinante. Contudo, estes resultados confirmam a hipótese 1.2.

A linhagem *35S:CKX2* foi mais susceptível à colonização por *G. intraradices* do a selvagem, e a presença de *P. fluorescens* resultou em maior percentagem de micorrização, independentemente da linhagem ou fertilização, sugerindo que esta bactéria é uma auxiliar da micorrização no tabaco. Estes resultados confirmam as hipóteses 2.1 e 2.2.

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Plant beneficial soil microbes

Introduction

The rhizosphere, the soil that is influenced by root activity, is considered one of the most fascinating hot spots of microbial activity and diversity (Jones & Hinsinger 2008) and an important zone in terms of defining the quality and quantity of the human terrestrial food resource (Hinsinger *et al.* 2009). Modern agriculture feeds about 6 billion people (Tilman *et al.* 2002) but the impacts on natural resources are a major global concern (Spiertz 2010). In the past 40 years, global cereal production has doubled, mainly due to increased yields resulting from greater inputs of fertilizer, water and pesticides, new crop strains, and other technologies of the "Green Revolution"(Tilman *et al.* 2001), increasing the global per capita food supply (Tilman *et al.* 2002). By 2050, global population is projected to be 50% larger than at present and global grain demand is projected to double (Alexandratos 1999). Doubling food production again, and sustaining food production at this level, are major challenges (Ruttan 1999). The expanding world populations will inevitably increase their demand on agricultural products, which will encompass with increasing pressure on land and water resources (Ryan *et al.* 2009). Although the "Green Revolution" has dramatically boosted food production its increases were accompanied by a depletion of groundwater and a drastic increase in nitrogen-based fertilizer usage, bringing environmental problems such as polluted waterways (Tilman *et al.* 2002). Controversially, the leaps in yield have still left many hungry and the revolution missed many developing nations, some of which have poor soils and are limited in access to irrigation or to expensive agrochemicals (Gewin 2010).

Current modern agriculture has high impute of fertilizers and low resource-use efficiency, especially for nitrogen (Spiertz 2010). Besides of being expensive, the high input of fertilizers combined with a low efficiency ultimately results in environmental problems such as soil degradation, eutrophication, pollution of groundwater, and emission of ammonia and greenhouse gases (Spiertz 2010). Furthermore, the intensive use of pesticides is harmful for non-target organisms in the ecosystem, including beneficial organisms, and poses a threat to human health. Moreover, the resurgence of pest populations can occur when their natural control is disrupted by pesticide application or when the target organism develops resistance (van der Werf 1996). In a near future, agriculture will have to meet at global level a rising demand for bio-based commodities such as food, feed, fiber and fuel, while satisfying even tighter constraints with respect to the safety of products and the environment (Spiertz 2010).

There is a growing insight that the key for a sustainable agriculture, one that does not depend on expensive inputs, may be within the rhizosphere and scientists are starting to see roots as central to their efforts to produce crops with a better yield (Gewin 2010). The intimacy of the interface between plants roots and their environment are essential for plant acquisition of water and nutrients and for beneficial interactions with soil-borne microorganisms, and yet, this same intimacy increases the vulnerability of plants to a range of biotic and abiotic stresses (Ryan *et al.* 2009). Engineering and management of the rhizosphere has been suggested as fundamental tool for the future of sustainable agriculture (Ryan *et al.* 2009), a tool that may ultimately reduce our reliance on agrochemicals by replacing their functions with beneficial microbes, biodegradable biostimulants or transgenic plants (Ryan *et al.* 2009; Gewin 2010). In natural ecosystems, plants have evolved a variety of strategies to modify the rhizosphere to reduce the impact of environmental stresses. The understanding of the processes involved may suggest ways in which the rhizosphere can be manipulated to improve plant health and productivity (Ryan *et al.* 2009). There is great potential to use the wide genotypic and agronomically induced diversity of root systems and their exuded chemicals to influence rhizosphere biology to benefit crops (Watt *et al.* 2006) as well as to harness beneficial microbes that grow on and around the rhizosphere that may improve crop yields (Gewin 2010).

Harnessing soil microbes is still a concept on its early stages and it is not clear whether, for example, introducing a new pathogen-fighting gene into a microbe or a new beneficial microbe into a poorly understood microbial community will be feasible approaches (Gewin 2010). Soil microbial communities are complex and contain numerous microbes that might have functional roles unknown to science and difficult to manage. A single gram has been estimated to contain about 10^7-10^{12} bacteria, 10^4 protozoa, 10^4 nematodes and 5-25 km of fungal hyphae (Young & Crawford 2004). Nevertheless, the improvement of our understanding of the rhizosphere has already allowed the identification of some examples of major agronomic effects explicable in terms of interactions between roots and soil microorganisms. As an example, Watt *et al.* (2003) demonstrated that reduced vigor of direct-drilled wheat was related to the inhibitory activity of *Pseudomonas*. In this example, the *Pseudomonas* build-up around the root tip due to the slow rate of root growth with deleterious effects on the plants development. This finding provided opportunities to explore other management and genetic options to increase the rate of root growth to avoid the problem (Watt *et al.* 2005), and highlight the importance of examine the interactions between the soil biology, the soil structure, and the patterns of root growth, in determining plant responses (Watt *et al.* 2006). In the following chapter I will conduct a review on the up to date

knowledge on mechanisms by which soil microorganisms are known to promote plant growth, with focus on two important groups that will be the aim in the present study.

Plant growth promoting rhizobacteria

The interaction between plant roots and the bacteria colonizing the rhizosphere, including *Pseudomonas*, may be beneficial for plants and can promote their growth (Osullivan & Ogara 1992; Glick 1995; van Loon *et al.* 1998). These bacteria are generally designated as plant growth-promoting rhizobacteria (PGPR) and their mechanism for growth promotion can be direct, e.g. enhancement of plant growth in the absence of pathogens by biofertilization, stimulation of root growth, or plant stress control, or indirect by suppressing plant pathogens through antibiosis, induction of plant systemic resistance (Lugtenberg and Kamilova 2009), or by stimulating plant symbiotic association with other soil microorganisms.

Improved nutrient availability

Biofertilization is a recently coined term which most commonly refers to the use of soil microorganisms to increase the availability and uptake of mineral nutrients for plants (Vessey 2003). Several "biofertilizer bacteria" have been identified so far. These include N_2-fixing bacteria such as *Rhizobium* and *Bradyrhizobium* which form symbiotic nodules in the roots of leguminous plants where they convert N_2 into ammonia used by the plant as a nitrogen source in exchange for photosynthetically fixed carbon (Vanrhijn & Vanderleyden 1995). Frankia bacteria, which form nodules in the roots of actinorhizal plants, also convert N_2 into ammonium making it available to the plant (Franche *et al.* 2009). Free-living N_2-fixing bacteria, such as *Azospirillum*, which have wide ecological distribution, can colonize the root surface of a wide diversity of plants, including those of agronomic importance such as wheat, rice, sorghum and maize and several non-gramineous crop species (Franche *et al.* 2009). Others, generally designated as phosphate solubilizing bacteria, including the genera *Pseudomonas, Bacillus* and *Rhizobium*, release nonspecific phosphatases, phytases, phosphonatases, and C-P lyases into the soil that liberate phosphorus from either organic or inorganic bound phosphates, thereby facilitating the plant uptake and promoting the yield of several important crops (Rodriguez & Fraga 1999).

Alteration of plant hormones

Rhizobacteria can also stimulate directly plant growth by synthesizing phytohormones from root exudates. The bacterial production of auxin, which can be synthesized from the exudated amino acid tryptophan, is one mechanism by which PGPR stimulates growth. Plant exudates concentration of tryptophan differs among plant species and this has been associated to different susceptibility to growth stimulation by *P. fluorescens* WCS365, one of the most well studied PGPR (Lugtenberg & Kamilova

2009). Some N_2-fixing bacteria have been shown to stimulate plant growth mostly by producing growth factors such as auxin, gibberellins, and cytokinins, rather than nitrogen fixation (Lugtenberg & Kamilova 2009). Others, such as *Pseudomonas fluorescens*, positively alter the root architecture and consequently increase plant nutrient up-take and growth (Gamalero *et al.* 2004).

Some rhizobacteria can control stress responses of plant roots (Kang *et al.* 2010). Fertilizers not taken up by crops can accumulate in deleterious concentrations and become soil contaminants that inhibit plant growth, most likely by activating plant stress responses such as increased ethylene production (Kang *et al.* 2010). Removal of soil contaminants such as fertilizer by some rhizobacteria can result in plant growth promotion. Additionally, the inhibition of plant growth caused by increased ethylene content in the plant can be overcome by the enzymatic activity of some PGPR, which hydrolyze 1-aminocyclopropane-1-carboxylate (ACC), the precursor of ethylene, to obtain energy for their own metabolism and growth (Kang *et al.* 2010).

Interaction with other soil microorganism

The indirect action of PGPR is done by suppressing plant diseases that inhibit plant growth. Soils in which a pathogen causes disease symptoms are called conducive soils, and spontaneous control of plant diseases by bacteria was discovered at several field sites around the world. Some soils, called suppressive soils, contain bacteria that protect plants against fungal diseases despite the presence of disease-causing pathogens in soil. Mixing small amounts of suppressive soil with large amounts of conductive soil makes the later suppressive (Lugtenberg & Kamilova 2009). One mechanism is the bacterial production of antibiotics, which kill the pathogen. To successfully perform this function, the antibiotic producing rhizobacteria must compete efficiently with other microbes for nutrients, be able to develop in the right microniche in the root, and should escape in sufficient numbers from predators feeding on rhizobacteria (Lugtenberg & Kamilova 2009). Other mechanism by which rhizobacteria may contribute for pathogen suppression is by signaling interference. Some rhizobacteria are able to degrade important signaling compounds required for pathogen synthesis of cell-wall-degrading enzymes and biofilm formation (Lugtenberg & Kamilova 2009), by which they restricting the pathogen development.

Interaction of some bacteria with the plant roots, even in minute extension, can result in plant resistance to some pathogenic bacteria, fungi, or viruses. This phenomenon, dependent on jasmonic acid and ethylene signaling in the plant, is called induced systemic resistance (ISR) and has been suggested to share many properties with innate immunity in humans (Lugtenberg & Kamilova 2009). ISR

differs from the systemic acquired resistance (SAR) because in the ISR the inducer does not cause disease while in the SAR the resistance is induced by previous contact with a pathogen. ISR was discovered by observing that resistance in plants could be induced by the rhizobacterium *Pseudomonas* sp. strain WCS417r against foliar pathogen *Fusarium* wilt of carnation (Vanpeer *et al.* 1991) and by selected rhizobacteria against the fungus *Colletotrichum orbiculare* in cucumber (Gang *et al.* 1991). In contrast to many biocontrol mechanisms, ISR by rhizobacteria does not require extensive root colonization and is strongly associated with induction by specific compounds produced by bacteria (Lugtenberg & Kamilova 2009). Plant inoculation with these compounds can suppress plant pathogens not only in the root but also in the foliar tissues.

Ultimately, rhizobacteria can also promote plant growth by stimulating root colonization by other beneficial microbes such as mycorrhizal fungi (Frey-Klett *et al.* 2007). These are generally designated as mycorrhizal helper bacteria (MHB), which include strains from many bacterial groups and genera and have been tested in many model plants, including herbaceous and woody plant species, mainly from temperate ecosystems (Frey-Klett *et al.* 2007). Gamalero *et al.* (2004) reported that the effect of a PGPR on tomato growth was synergistically increased by co-inoculation with arbuscular mycorrhizal fungi (AMF). This synergistic effect was associated with a positive effect of the PGPR on the root colonization by AMF (Gamalero *et al.* 2004).

Arbuscular mycorrhizal fungi

Among the beneficial soil microbes that colonize the root are the mycorrzizal fungi. At least five types of mycorrhizas are recognized and reviewed elsewhere (Smith & Read 2008). The most wide spread are the arbuscular mycorrhizal fungi (AMF), which form functionally important symbioses with species from many terrestrial plant families and are completely reliant on their hosts to obtain photosynthetically fixed carbon (C) (Smith & Read 2008). All AMF species are included in the phylum *Glomeromycota* which is currently divided into four orders, with most described species belonging to the Glomerales and Diversisporales. Three families have been described within the Diversisporales; of these, the Acaulosporaceae and Gigasporaceae contain the greatest number of described species. The Glomerales consists of a single family (Glomeraceae) with a single genus (*Glomus*) (Powell *et al.* 2009). Plant fossils records from the early Devonian Rhynie Chert (400 Myr) revealed arbuscules within fossil roots (Redecker 2002), a fungal structure belonging to the Glomeromycota. More recently, phylogenetic analyses revealed that the presence of three plant genes required for mycorrhizal formation in rice were present in nearly all major plant lineages, and that these genes were both vertically inherited and functionally conserved during the evolution of land plants (Wang *et al.* 2010). Wang et al. (2010) suggested that plant-mycorrhizal fungus symbiosis was one of the key processes that contributed to the origin of land flora.

It is well recognized that AMF have a pivotal involvement in plant mineral nutrition in terrestrial ecosystems (Rillig 2004). Since primary production in the majority of the ecosystems is limited by belowground resource availability, AMF has been described as keystone mutualists in these ecosystems (Rillig 2004). The largest effect of AMF on plants is on phosphorus (P) nutrition. Besides of being required by both plants and AMF in relative large amounts, P is poorly mobile in the soil and occurs in very low concentration in the soil solution (Smith & Read 2008). There is also convincing evidence for increased AMF uptake of zinc, calcium, cooper, and nitrogen (Smith & Read 2008). Additionally to nutrient uptake, AMF may improve the water uptake of drought stressed plants.

Improved nutrient uptake

Colonization of plant roots by AMF is achieved via spores and mycelia originating from infected roots or soil. The hyphae enter the root tissues and colonize the cortex by developing intercellularly running hyphae, and intracellularly forming hyphae, coils and arbuscules. AMF also develop an external mycelium which ensures access to soil-derived nutrients, from which some are transferred to the root.

The arbuscules and coils are thought to be the main interfaces for the exchange of mineral nutrients in return for photosynthetic fixed C (Smith & Read 2008). As obligate symbionts, the mutualism advantages for the fungi are immense. More stable physiological conditions (e.g. water potential, solute concentrations, pH) in the root apoplast compared with the soil environment may be an advantage for the fungi, besides the organic C supply. Plants can take up nutrient by direct root uptake from soil or via an AM fungal symbiont. However, the fungal mycelium in the soil can absorb nutrients beyond the nutrient depletion zone, increasing the effectiveness with which the soil volume is exploited. Additionally, the soil pores that can be penetrated by the hyphae are perhaps an order of magnitude smaller than those available to roots, making AM roots often more efficient in nutrient acquisition, per unit length, than non-mycorrhizal roots (Smith & Read 2008). However, the AM colonization of roots and the involvement of the fungi in energy- and substrate requiring activities like nutrient uptake, vegetative growth and spore production, also represent a C cost for the plant. Nevertheless, the involvement of the fungi in nutrient uptake often increases the ability of the plant to fix CO_2 and, consequently, the expenses of the fungi is offset by a positive cost-benefit balance (Smith & Read 2008), explaining the competitive advantage of AM plants in ecosystems limited by belowground nutrient availability.

Alteration of plant hormones

Additionally to plant nutrient enrichment, plant growth may be affect directly by AMF via alteration of plant phytohormone content. Phytohormones such as cytokinin (CK), ethylene (ET), jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), gibberellin (GA), and auxin have been suggested to participate in the communication between AMF and plant (Ludwig-Müller 2000). A particular interesting interaction of AMF with plant hormones is the alteration of plant CK, a phytohormone that regulates cell division and development (Werner *et al.* 2001). When cytokinin is reduced in the plant, shoot growth is reduced, while root formation is enhanced, leading to an enhanced root to shoot ratio (Werner *et al.* 2001), a trait associated with plant strategy for soil exploration and exploitation in nutrient deficient soils. Recent studies have shown that plant phosphorus starvation reduces the cytokinin content (Franco-Zorrilla *et al.* 2005)and that AM plants accumulate more CK than non-mycorrhizal plants in both shoots and roots (Hause *et al.* 2007). This is of particular interest because P availability in the soil is a major factor affecting the plant-mycorrhizal symbiosis (Smith & Read 2008).

In the case of ET, this phytohormone seems to be involved in the regulation the mycorrhization itself. Under high plant ET concentrations, both root and shoot growth and mycorrhization are reduced,

at low ET concentrations root length may still be reduced but not mycorrhization (Geil & Guinel 2002). The JA homeostasis within the plant seems to be also involved in the control of mycorrhization (Hause *et al.* 2007). JA application in low concentrations to plants stimulates mycorrhizal development, while in high concentration inhibits. Recent results revealed that in roots of mycorrhizal plants JA levels are increased in comparison to roots of non-mycorrhizal controls, implying a possible role of JA in AM formation (Hause *et al.* 2007). Altered SA accumulation in mycorrhizal plants indicated that SA might be also involved in the susceptibility of plants to AM fungi (Hause *et al.* 2007). The interaction of AMF with plant ABA content is not clear yet, however, it has been suggested that a reduction of ABA in leaves of AM plants may reflect the effects of mycorrhiza e.g. improving water relations of drought stressed plants (Ruiz-Lozano 2003). Additionally, AMF can interfere with GA balance, and it has been reported that the AM fungus *Glomus mosseae* is capable of synthesizing at least two gibberellin-like substances (Barea & Azconaguilar 1982). Concerning auxins, it has been suggested the existence of a complex control mechanism to regulate the levels of free and conjugated auxins in AM plants, which are induced during early stages of the formation of an AM symbiosis (Fitze *et al.* 2005).

Ultimately, AMF alteration of plant hormone content can result in changes the architecture of the host root system (Price *et al.* 1989; Yano *et al.* 1996; Paszkowski *et al.* 2002; Gamalero *et al.* 2004; Olah *et al.* 2005; Gutjahr *et al.* 2009). Gutjahr *et al.* (2009) showed that *Glomus intraradices* preferentially colonized the lateral root of rice and, additionally, AM colonization induced the formation of its preferred tissue for colonization. Olah *et al.* (2005) reported that the induction of lateral root formation can start before colonization take place by signaling exchange between AMF and plant.

Pathogen protection

Interestingly, the positive effects of AMF on root architecture, that can be translated in enhancement of root branching, is associated to higher susceptibility for pathogen attack (Newsham *et al.* 1995). However, plants with inherently highly branched root systems show fewer necroses in mycorrhizal compared to non-mycorrhizal plants (Norman *et al.* 1996). There is evidence that plants that take up more nutrients due to their AM fungal symbiont have an increased tolerance for pathogenic infections (Bodker *et al.* 1998; Karagiannidis *et al.* 2002), which is not necessarily a direct consequence of improved nutrition (Shaul *et al.* 1999; Fritz *et al.* 2006). Additionally, Maherali & Klironomos (2007) found taxonomic variation within the AM fungal phylum Glomeromycota, both in terms of nutrient responses and pathogen protection.

Functional trait divergences

The biggest functional contrast among AMF is noted between the families Gigasporaceae and Glomeraceae. Fungi from the first tend to extensively colonize soil while exhibiting slow and limited colonization of roots, while fungi from the later rapidly and extensively colonized host root but produced limited biomass in soil (Hart & Reader 2002). Furthermore, these functional differences have been hypothesized to be related to specific mechanisms for promoting host plant growth (Maherali & Klironomos 2007) with Glomeraceae offering higher pathogen protection and Gigasporaceae contributing more for nutrient and water uptake (Newsham *et al.* 1995; Maherali & Klironomos 2007). Powell *et al.* (2009) studied the evolution of functional and life-history traits among AMF families, and found no evidence for a trade-off in fungal biomass allocation between root vs soil colonization; rather these traits were positively correlated in their experiments. However, they have found enough evidence for evolution of fungal colonization strategies and functional benefits of the symbiosis to host plants. AMF with increased soil colonization are positively correlated with total plant biomass and shoot phosphorus content, suggesting that a longer hyphal network in the soil offers higher nutrient up-take efficiency and consequent higher promotion of plant growth. Although the effect of AM fungi on infection by root pathogens was phylogenetically conserved, they detected no evidences for coevolution between the extent of AM fungal root colonization and pathogen infection.

A practical example presented by Ryan *et al.* (2005) showed that AMF colonization in wheat and field pea did not increased nutrient uptake, biomass, or yield during winter in spite of a strong P limitation. They concluded that high colonization by AMF was unimportant for the productivity of these crops and that AMF were likely parasitic under those circumstances. It is well recognized that in natural habitats AMF have a pivotal involvement in the improvement of plant fitness, however, in systems managed by humans this is not always the case (Johnson *et al.* 1997). Mycorrhizal associations with crops can become parasitic on the plants when net cost of the symbiosis exceeds net benefits (Johnson *et al.* 1997). Maherali & Klironomos (2007) findings also suggests that the composition of AMF assembly in agricultural soils might be of importance to determine net benefits of the symbiosis, depending on which functional traits of the symbiosis are more relevant for crop productivity.

Proposed study

To gain further insights into the interactions between plant growth, soil biota and soil nutrient availability, I will focus in this study the effects of interaction between *Glomus intraradices*, *P. fluorescens*, soil P and plant endogenous CK content on tobacco growth.

Introduction

Plant growth in relation to mineral nutrient limitations depends much on the capacity of roots to access and mediate the availability of essential nutrient in soil (Darrah 1993). To effectively exploit soil nutrients, plants modify to some extent their root growth and morphology along with the development of a rhizosphere (Lynch 1995). N and P are key nutrients for plants in many sustainable agricultural systems across the globe (Tilman *et al.* 2002), and differ in their mobility and available concentration in soil. N is present in relative higher concentrations (NH_4^+) and has a greater diffusion coefficient (NO_3^-), moving more freely toward the root through mass flow. Therefore, a root system size (relative to the shoot growth) and morphology that facilitate water uptake (thicker roots) are of greater relative significance to N nutrition (Richardson *et al.* 2009). On the other hand, P is present at low concentrations in soil solution and has poor diffusivity (either has HPO_4^{2-} or $H_2PO_4^-$), and generally the region around the roots are quickly depleted from P. A rapid rate of root elongation, high root to shoot biomass ratio, increased root branching for better exploration, high specific root length (i.e. length per unit mass), as well as the presence of root hairs, release of root exudates and/or association with soil biota are of particular importance for P uptake (Richardson *et al.* 2009).

Plants appear to accommodate a trade-off between thicker roots (which allow greater capacity for water transport) and fine roots (associated with greater soil exploration) by exhibiting plasticity in root diameter (and morphology) according to the environmental conditions (Richardson *et al.* 2009). This root "foraging" capability is considered to be an important plant response to optimize resource allocation in regard to N and P capture and is of particular ecological importance (Hodge *et al.* 2009).

Objective 1 - Root morphology and plant growth

Soil microbes can play an important role in the "foraging" activity of root, affecting root morphology, nutrient uptake and plant growth (Smith & Read 2008; Lugtenberg & Kamilova 2009; Richardson *et al.* 2009). For example, enhanced plant growth by *Glomus* sp. and *P. fluorescens* has been associated with positive but somewhat distinct effects on root morphology (Gamalero *et al.* 2004). While *Glomus* sp. increased root surface area and volume, number of tips and degree of root branching, *P. fluorescens* increased root length, total root surface area and volume. Additionally, synergistic effects between *Glomus* sp. and *P. fluorescens* on plant growth were associated with accumulative effects on root morphological parameters (Gamalero *et al.* 2004). Previously, Gamalero *et al.* (2002) reported that soil nutrient availability was a factor conditioning the effects of both *Glomus* sp. and *P. fluorescens* on root morphology, suggesting the modification of root morphogenesis by *Glomus* sp. was associated to improved plant nutrition, while by *P. fluorescens* was associated to its ability to synthesize auxin (as demonstrated in vitro).

Auxin and CK have major but contrasting roles in root morphogenesis and development (Chapman & Estelle 2009; Hodge *et al.* 2009), and are highly responsive to N and P availability (Walch-Liu *et al.* 2005). *P. fluorescens* effects on plant growth and root morphology has been suggested to be associated with interference on plant hormonal balances via direct synthesis (de Salamone *et al.* 2001; Gamalero *et al.* 2004). AMF have been suggested to interfere directly on the plant hormonal balances (Shaul-Keinan *et al.* 2002) or indirectly by enhancing nutrient uptake (Barker & Tagu 2000). The use of tobacco mutant with endogenous reduced levels of CK grown on different P amendments, and its comparison to a wild type grown under the same P amendments, will allow us to understand better the mechanisms, direct or indirect, by which these soil microbes can affect root morphology, nutrient uptake and, consequently, plant growth. In particular, I will test the hypothesis 1.1 and 1.2.

Hypotheses

1.1. The effects of *G. intraradices* or *P. fluorescens* on tobacco growth are associated with the alteration of root morphology.

1.2. The effects of *G. intraradices* or *P. fluorescens* on tobacco root morphology depend on soil P availability and plant endogenous CK content.

Objective 2 - Mycorrhization

Higher P availability in the soil often decreases AMF root colonization. By contrasts, when the plant is growing on P deficient soils, the AMF colonization is enhanced (Smith & Read 2008). This suggests that plants might have a mechanism that can regulate mycorrhization dependent on P starvation, which is a plant stress associate to reduced CK content (Franco-Zorrilla *et al.* 2005). It is plausible that low CK levels in the host plant are favorable to the establishment of the mycorrhizal symbiosis. The important signaling role of CK in P sensing and in the control of plant metabolism and development may also be associated with the mediation of root AMF colonization. To answer this question I will test the hypothesis 2.1.

Many *Pseudomonas* sp. have been reported to be mycorrhizal helper bacteria (MHB) on several mycorrhizal fungi, a trait that has been considered fungal species specific (as for bacterial antagonism against pathogenic fungi) (Garbaye & Duponnois 1993; Frey-Klett *et al.* 2007). *Pseudomonas* can enhance mycorrhiza formation by some fungi but inhibits the establishment of symbiosis by others (Garbaye & Duponnois 1993). The mechanism for fungal species specificity has been suggested to be related with the segregation of antibiotic compounds that may affect negatively some fungal species but not the others (Frey-Klett *et al.* 2007), a similar mechanisms by which *P. fluorescens* can suppress root pathogenic fungi (Lugtenberg & Kamilova 2009). In the case of AMF symbiosis, several examples of MHB have been identified (Frey-Klett *et al.* 2007). However, to the extent of my knowledge, until now no study reported enhancement of AMF colonization by a rhizobacteria isolated from a non-mycorrhizosphere. The use of a *P. fluorescens* isolated from such rhizosphere, known to affect root pathogenic fungi, can potentially allow us to identify a new source of MHB. This question will be answer by testing hypothesis 2.2.

Hypotheses

2.1. *G. intraradices* colonization of tobacco roots with reduced endogenous CK content is enhanced in comparison to its wild type.

2.2. A bacteria isolated from a non-mycorhizoshpere can enhance *G. intraradices* colonization of tobacco roots.

Materials and methods

Microorganisms and plant lines

The *Pseudomonas fluorescens* Migula strain 8569 was obtained from the German Resource Centre for Biological Material (DSMZ). This strain was originally isolated in Germany from the rhizosphere of *Brassica napus*, a non-mycorrhizal plant species from the family Brassicaceae, and is cataloged by DSMZ as inhibitor of the plant pathogens *Fusarium culmorum, Rhizoctonia solani, Sclerotina sclerotiorum* and *Verticillium dahliae*. Additionally, it has been reported to be a PGPR on sugar beet by antagonism against the root pathogens *R. solani*, *Pythium ultimum* and *Pythium debarianum* (Kristek *et al.* 2008).

The arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith strain MUCL 43204 was obtained from Glomeromycota In vitro Collection (GINCO), Belgium. This fungus was originally isolated from a meadow in the province of Ontario, Canada. The fungus species *Glomus intraradices* have been previously reported to colonize the root of *Nicotiana tabacum* L. cv. Samsun NN (Maier *et al.* 2000; Medina *et al.* 2003):

The two plant lines of tobacco (*Nicotiana tabacum* L. cv. Samsun NN), the wild type and a transgenic line *35S:CKX2*, were kindly supplied by Prof Schmülling from the Plant Genetics Department, Freie Universität Berlin. The construction of the transgenic tobacco line *35S:CKX2* was reported by Werner et al (2001) and consisted in cloning the *AtCKX2* gene from *Arabidopsis thaliana*, a gene encoding a protein with cytokinin oxidase (*CKX*) activity. They genetically transformed the tobacco wild type by positioning this gene under the control of a constitutive 35S promoter, resulting in a transgenic line *35S:CKX2* with overexpression of cytokinin oxidase and, consequently, reduced endogenous concentrations of different cytokinin metabolites. The cytokinin-deficient plants from the *35S:CKX2*-expessing transgenic line develop stunted shoots with smaller apical meristems compared with the wild type, while root meristems are enlarged and give rise to faster growing and more branched roots, resulting in increased root to shoot ratio (Werner *et al.* 2001).

Preparation of the treatments

Preparation of Pseudomonas fluorescens inoculum

P. fluorescens was supplied as dried culture sealed under vacuum in a double vial preparation. The vial was opened in sterile conditions at the clean bench by heating the ampoule tip in a flame and cracking it with sterile water drops. Using flamed sterilized forceps, the insulation material was removed, the inner vial collected and the cotton plug on the inner vial detached. The top of the inner vial was flamed to assure decontamination. The bacteria pellet inside the inner vial was rehydrated during 30 minutes by adding 0,5 ml of liquid Lysogeny broth (LB) medium (Luria/Miller) (Roth, X968.2), previously sterilized by autoclave (20 min at 121°C). The inner vial content was gently mixed using an inoculation loop sterilized by flame. Several aliquots from the vial content were transferred with the loop into Petri dishes containing Caso Agar medium (Roth, X937.1), previously autoclaved (121°C for 20 minutes). The Petri dishes were sealed with parafilm and cultures were amplified in the incubator at 26°C for 3 days.

Several Petri dishes were prepared with Caso Agar medium supplemented with rifampicin (Sigma, R3501) in a stepwise concentration method (20, 40, 60, 80 and 100 mg ml^{-1}). Rifampicin was added into the medium as a dimetilsulfoxid (DMSO; Sigma, 41640) solution, with a rifampicin concentration of 50 mg ml^{-1} DMSO. The rifampicin solution was sterilized with a syringe driven filter unit (0,22 µm) at the clean bench wile pouring it into the warm (50°C) liquid medium and mixed before solidification. To growth the bacteria on the medium, an aliquot from one amplified bacteria culture was transferred into the first rifampicin stepwise concentration (20 mg ml^{-1}), and cultured in the incubator for 3 days, after which a similar step was done for the following stepwise concentrations until obtaining a spontaneously resistant mutant able to grow on rifampicin supplemented medium (100 mg ml^{-1}).

Rifampicin has antibiotic activity against a wide range of microorganisms especially against bacteria, by inhibiting bacterial DNA-dependent RNA polymerase (the enzyme responsible for DNA transcription) due to the formation of a stable enzyme-drug complex with the ß-subunit of RNA polymerase (RNAP-Rif), suppressing the initiation of chain formation in RNA synthesis (Sigma R3501 - product information sheet). Bacteria can develop resistance to rifampicin by mutations due to changes of the structure of the ß-subunit of RNA polymerase. Rifampicin is also active against some viruses and has antifungal activity probably due to some other mechanism of action than inhibition of a fungal RNA polymerase (further information online: Sigma R3501 - product information sheet). The use of a rifampicin resistance *P.*

fluorescens mutant allows the post-harvest selective culture of the target bacteria, by inoculating the selective media with solutions from root surface extractions.

The selected rifampicin resistant mutant was amplified in the incubator for 3 days on Petri dishes with rifampicin enriched Caso Agar (100 mg ml^{-1}). After 3 days of growth, the bacteria were scraped from the medium and suspended in a sterile buffer solution (0.1 M $MgSO_4$. $7H_2O$). The bacteria density of the suspension was determined by using stepwise dilutions and counting bacteria with a hemocytometer under the microscope. Density of the suspension was adjusted to approximately $6x10^9$ colony forming units (CFU) per ml of suspension, by adding sterile buffer solution. From the adjusted bacteria suspension, 1 ml aliquots was used to inoculate the tobacco plants assigned to *P. fluorescens* treatments. Half of the bacteria suspension was autoclaved (20 min at 121°C) to produce a control solution, from which also 1 ml aliquots were used to inoculate the tobacco plants assigned to the non-bacteria control. All step concerning bacterial transfers and dilutions were done in aseptic environment at the clean bench, and the bottles containing the bacteria inoculum or control solution were opened out of sterile environment for the first time at the greenhouse, before inoculations.

Preparation of Glomus intraradices inoculum

The in vitro fungal inoculum of *G. intraradices* was supplied inside vials containing fragments of Ri T-DNA transformed carrot root heavily colonized by the fungus and embedded in the gel of a modified Strullu-Romand (MSR) medium (Declerck *et al.* 1998). The in vitro system provides aseptic mono-specific fungal inoculums consisting of spores, hyphae fragments and roots heavily colonized, and each vial contains between 500 and 1000 spores (personal comments of Dr Sylvie Cranenbrouck, GINCO).

13 vials were opened in aseptic environment at the clean beach and the medium of each vial was collected into a sterile centrifuge tube (15 ml). The MSR medium, in which the fungal inoculums and roots were embedded, was dissolved by adding 2 ml of 10 mM sodium citrate into each centrifuge tubes using a sterile syringe driven filter unit (0,22 µm). The tubes were closed, removed from the aseptic environment and incubated on the shaker for 20 minutes to dissolve completely the medium gel. After shaking very well, the tubes were brought again into the aseptic environment and the entire tube content was passed through a 38 µm sieve, previously autoclaved. The spores as well as the clumped roots in the sieve were rinsed with sterile deionized water several times to wash away the dissolved medium and the sodium citrate, then recollected into tubes and filled up to 10 ml with sterile deionized water and stored in 4°C for 2 day, allowing the fungal inoculums to precipitate to the bottom. The

precipitation of the inocula was observed at the stereomicroscope one day after storage at 4°C. To produce a control solution for the non-mycorrhizal treatments, the tubes were carried into the clean beach and half of the solution (5 ml) of each tube was collected with a syringe and passed into new sterile tubes using a syringe driven filter unit (0,22 µm). It is assumed that the inoculum solution may contain organic compounds apart from the fugal inoculum that can affect plant growth, and this procedure assures that the same amount of organic compounds are added to all experimental treatments, but not the AMF, which is prevented to enter the control solution by the 0,22 µm filter. The tubes containing fungal inoculum solution (5 ml each) were vortex for 40 min to detach spores from hyphae and roots. The clumped roots of each tube were aseptically excised in small fragments and distributed into 5 sterile eppendorf vials (2 ml). The spores and hyphae solution in each tube were evenly distributed between the 5 eppendorf vials, by vortexing the tube solution to homogenise and transferring 1 ml with a micropipette into a vial. The tube was vortexed again before the next transfer, to produce a homogeneous solution among vials. Each vial represents the inoculum source for a seedling assigned to *G. intraradices* treatment and is estimated to have between 100 and 200 spores besides loosen hyphal fragments and colonized root fragment. The fungus *G. intraradices* is known to colonize plant host from this three sources of inoculum (Smith & Read 2008).

Preparation of modified Hoagland's solution

Two modified Hoagland's solutions were produced to supplement the plants with low or high P amendments (8 and 16 mg L^{-1}, respectively). Initially, four macronutrients stock solutions (calcium nitrate, potassium nitrate, monopotassium phosphate and magnesium sulfate) and two micronutrient stock solutions (micronutrient mix and iron) were prepared by adding hydrated or pure salt into distilled water according to Table 1. Each nutrient solution was prepared with half strength by mixing specific volumes (Table 1) of each stock solution in a 1 L beaker with 500 ml of distilled water. The beaker was refilled until 800 ml and the solution was mixed in a magnetic bar stirrer for 5 min, after which it was transferred into a plastic can and refilled with distilled water until 10 L. The stock solutions were maintained in the fridge at 4 °C when not in use. Macronutrient concentration in both modified Hoagland's solutions were adapted from Douds and Schenck (1990), micronutrient followed the original Hoagland's solution (Hoagland & Arnon 1950), and the iron supplement was take as recommended by Smith *et al.* (1983). The volume of nutrient solution added to each tobacco seedling was adapted from Smith *et al.* (1983).

Table 1. Stock solutions for macronutrients and micronutrients used to produce 10L of modified Hoagland's solution with two phosphorus levels (8 and 16 mg L^{-1}).

Macronutrients (8 P)	**g mol^{-1}**	**mg ml^{-1}**	**Stock (mM)**	**Stock added (ml/10L)**	**Salt in solution (mg/10L)**
$Ca(NO_3)_2.4H_2O$	236,2	236,2	1000	27,6	6519,1
KNO_3	101,1	101,1	1000	30,0	3033,0
KH_2PO_4	136,1	136,1	1000	2,5	340,3
$MgSO_4.7H_2O$	246,5	246,5	1000	10,0	2465,0

Macronutrients (16 P)	**g mol^{-1}**	**mg ml^{-1}**	**Stock (mM)**	**Stock added (ml/10L)**	**Salt in solution (mg/10L)**
$Ca(NO_3)_2.4H_2O$	236,2	236,2	1000	34,4	8125,3
KNO_3	101,1	101,1	1000	25,0	2527,5
KH_2PO_4	136,1	136,1	1000	5,0	680,5
$MgSO_4.7H_2O$	246,5	246,5	1000	10,0	2465,0

Micronutrients (8 and 16 P)	**g mol^{-1}**	**mg ml^{-1}**	**Stock (mM)**	**Stock added (ml/10L)**	**Salt in solution (mg/10L)**
H_3BO_3	61,8	2,86	46,25	5,0	14,30
$MnCl_2.4H_2O$	197,9	1,81	9,15		9,05
$ZnSO_4.7H_2O$	287,6	0,22	0,76		1,10
$CuSO_4.5H_2O$	249,7	0,08	0,32		0,40
$Na_2MoO_4.2H_2O$	242,0	0,03	0,14		0,15

Iron (8 and 16 P)	**g mol^{-1}**	**mg ml^{-1}**	**Stock (mM)**	**Stock added (ml/10L)**	**Salt in solution (mg/10L)**
FeIII EDTA	367,1	0,06	0,18	5,0	0,3

Treatments and plant growth conditions

A full factorial experiment with a randomized block design was set up at the greenhouse, with presence or absence of *G. intraradices* or *P. fluorescens*, with low or high P amendment, and with the tobacco wild type or the transgenic *35SCKX2* line, in a total of 16 treatments, each one with 8 replicates.

500 ml containers were sterilized in bleach bath for 20 minutes and washed with tap water. A cotton mesh was inserted into the bottom of each container to prevent substrate losses. Sand (CEMEX GmbH, Kraatz, Germany) was autoclaved at 121°C for 20 minutes and distributed into each container. Seeds from the tobacco wild type and *35S:CKX2* line were surface sterilized with a saturated 1.2% sodium hypochlorite solution in a microcentrifuge tube incubated for 5 min on an orbital shaker. After incubation, seeds were washed with sterile water 3 times by brief centrifugation. Seeds were air dried on sterile Whatman paper at the clean beach and placed on MS media plates. Seedlings were grown on MS medium for two weeks at 25°C with 16 h white light cycle (125µE).

128 containers filled with sand were place in the greenhouse (16 h light and 23°/28°C night/day temperature). A central hole was made with a dibber on the sand surface of each container. 64

containers received 1 ml each of *G. intraradices* inoculum solution by collecting the inoculum from the eppendorf tubes with a micropipette and dripping it into the hole in the sand. The other 64 containers received 1 ml each of the *G. intraradices* control solution, to establish the non-mycorrhizal controls. The two week tobacco seedlings were then transplanted from the MS medium into the holes, carefully accommodating the roots in the sand. Tobacco lines were crossed with *G. intraradices* treatments. 34 seedlings of each plant line were assigned to *G. intraradices* treatment and the non-mycorrhizal controls, respectively. Seedlings were covered with a transparent plastic cover to maintain the air moisture around the leaves during first days at the greenhouse.

After two days of transplanting, 16 seedlings of each plant line assigned to the mycorrhizal treatment or to the non-mycorrhizal control received 1 ml of *P. fluorescens* inoculum, by collecting the bacterial suspension with a micropipette and dripping it around each seedling according to treatment. In a similar manner, 1 ml of bacteria control solution was added to the non-bacteria controls. The containers were carefully watered with tap water to saturation without dripping, to encourage bacterial dispersion in the sand substrate. During the initial four days after transplantation the moisture in the soil was regularly checked and maintained. After four days, the transparent plastic cover was removed and thereon seedlings were watered every second day.

After one week of transplantation the watering was replaced by supplementation with the modified Hoagland's solutions every second day and distilled water two times a week. 8 seedlings from each plant line assigned to a mycorrhizal or a bacteria treatment received a high P amendment (16 mg ml^{-1}) or low P amendment (8 mg ml^{-1}). The amendments consisted in 30 ml of each nutrient solution every second day. Six weeks after transplantation the seedling mortality was too high and it was decided to install a new setup using the same containers and microbial inocula. It was hypothesized that the stress due to the transplantation from the nutrient rich MS medium into the poor sand substrate might have caused irreversible damage to the seedlings.

To install a new set-up, peat pellets were sterilized by autoclave at 121°C for 20 minutes. The fabric in the bottom of each peat pellets was cutoff using a sterilized scissor, and one peat pellet was gently inserted at the sand surface of each container. The whole system was watered to keep the peat pellets well moist. Seeds from both tobacco lines were surface sterilized as before, after which three seeds of each plant line were sown onto a peat pellet according to treatment assignment, repeating the full factorial randomized block design. The germinating seeds were covered with a transparent plastic

cover to maintain air moisture during the early stages. Containers were watered every day in the first week and thereon every second day. After three weeks of germination the plastic cover was removed and the nutrient amendment was initiated. Each container received 30 ml of nutrient solution every second day and watered twice a week with distilled water. The width of the biggest leaf in the biggest seedling in each container was measured regularly in the initial phase, and when reached more than 20 mm, the seedlings in that container were thinned to one. Plants were harvested two months after sowing.

Harvesting

Plants were harvested by cutting the shoot of all seedlings at ground level. The shoot fresh weight was determined with a precision balance, and shoots were stored in plastic bags at 4°C until further use. To harvest roots, the peat pellet was separated from the sand substrate by carefully cutting roots links between the peat pellet and the sand. The diameter and height of each peat pellet was measured to determine its volume. Peat pellets were then carefully opened to collect the roots within, which required a careful separation and washing of roots. The rest of the sand substrate was gently poured onto a tray, and roots were carefully separated and collected from the sand. The fresh weights of root portions were then determined with a precision balance and the roots stored in plastic bags at 4°C until further use.

To determine plant dry mass, the shoots were placed in the oven at 40°C for a week, after which dry weights were measured. After determining root morphology, the roots were also placed in the oven at 40°C for a week, and weighted.

Parameters analyses

Determination of P. fluorescens colonization

The determination of *P. fluorescens* colonization of roots was conducted by collecting a fresh root aliquot from each plant during harvest and measuring its weight. The root aliquot was then placed into 15 ml centrifuge tube and filled with 10 ml of buffer solution (0.1 M $MgSO_4$. $7H_2O$). From each treatment a replicate was selected to determine the range of dilution required to produce a bacterial culture with a countable number of bacterial colonies, i.e. 30 to 300 CFUs per plate. The selected replicates were vortexed for 15 minutes to create a microbial suspension from the root pieces. Then, from each suspension a 10 stepwise dilution was prepared at the clean beach. An aliquot (200 µl) from each dilution was used to inoculate a plate with rifampicin enriched Caso Agar (100 mg ml^{-1}). Cultures were grown in the incubator at 26°C for 3 days, after which it was detected that the medium was not selective enough against fungi, preventing accurate *P. fluorescens* CFUs counting.

The recommended medium by DSMZ for the *P. fluorescens* is Caso Agar. However, a deeper search in the literature revealed a better option for isolation of *P. fluorescens*, the King Agar B medium (King *et al.* 1954). A similar step for the dilutions were repeated using King Agar B medium (Sigma, Fluka 60786), enriched with CFC selective supplement (Sigma, Fluka 53477) (0.07 mg ml^{-1}) and rifampicin (100mg ml^{-1}). The CFC selective supplement is an antibiotic supplement for the selective isolation of *Pseudomonas* species composed by 1:1:5 of Cetrimide, Fucidin and Cephaloridine. Additionally, King Agar B enhances the *P. fluorescens* elaboration of fluorescein which becomes fluorescent under UV light. The plates were then grown in the incubator for 3 days. However, this medium was still not selective enough against fungi, not enabling CFUs counting. Given the time passed since harvesting, and the difficulties to establish a proper selective medium, it was decided not to continue the determination of *P. fluorescens* root colonization. The method should have been conducted in short after harvesting because it is expected that the rhizobacteria populations will decrease drastically after some days of root death.

The method to determine bacterial root colonization was adapted from Gamalero et al (2004) and was used in the lab for the first time in this study. Some methodological details were only acknowledged during its application. The method was later improved by adding Cycloheximide solution (Sigma, C4859) to the King Agar B enriched with CFC selective supplement and rifampicin and applied in the lab with relative success in other experiment (data not shown).

Determination of mycorrhizal colonization

After determine root morphology and root dry weight, root pieces from each plant were used to determine AMF percentage of root colonization. The staining of the root followed an internal protocol in the lab adapted from the Ink & Vinegar method proposed by Vierheilig et al. (1998).

The root pieces were cut into fragments about 2 cm long, wrapped in perm paper and closed inside small plastic cassettes. The cassettes were placed in a beaker and covered with 10% KOH solution (100 g L^{-1} H_2O). The beaker was incubated in a water bath at 90°C for 10 minutes. The KOH solution was poured out retaining the cassettes, and roots were washed with tap water 3 times. The ink-vinegar staining solution (1:1:8 ink:vinegar:H_2O) was added into the beaker covering all cassettes and the beaker placed again in the water bath at 90°C for 10 minutes. After the staining, the ink-vinegar solution was poured off, retaining the cassettes. The beaker was filled with lactoglycerol and kept at room temperature overnight to allow excess stain to leach from roots.

The roots were taken out of the cassettes into a Petri dish using forceps. When there was enough root material, up to 20 root pieces were randomly selected and aligned on a slide and covered with a cover slip. The step was repeated for all root samples, washing carefully the Petri dish between to avoid cross contamination. The slides were stored in a slide box at 4°C until further use. Slides with root were examined under a microscope to note the presence of AMF structures. Percentage of colonization was determined dividing the number of AMF presences by the total number of observations.

Measurement of root morphology

Root morphological parameters were measured on scanned images from roots using WinRhizo software (Regent Instruments Inc). The fresh roots from each sample carefully washed from sand particles were spread on the WinRhizo tray (15x20 cm) and covered with water. The tray was place on the scanner (Epson perfection V700 photo) and an image from the root system was acquired using the default calibration method *Intrinsic*. The acquisition parameters consisted on resolution medium 400 with image grey levels, and the analysis on a pixels classification method based on automatic grey levels. The measured parameters consisted on total root length, average root diameter, total root surface area, total root volume and specific root length (total root length/root fresh weight). The later was calculated by dividing the total root length by the root fresh weight.

Determination of carbon and nitrogen content in the shoot

The dry shoots of each plant was cut in small pieces, spread in a layer on a paper sheet, mixed to homogenize before sub-samples were taken and put into an eppendorf tube. The material was cut again inside the eppendorf with a scissor. When the eppendorf was half full, a metal sphere was inserted and the eppendorf placed in the mill tray. Shoot samples were grounded to fine particles using a cyclone mill with a frequency of 25 for 5 minutes. 2-4 mg of each grounded sample was weighted into individual zink capsules. The zink capsules were then assembled on an autosampler of a CN Elemental Analyzer (Euro EA, HEKAtech GmbH, Germany), using acetanilide as standard (HEKAtech M.135.17). The Elemental Analyzer was run using standard procedures. The functional principle involves dynamic, spontaneous combustion with subsequent chromatographic separation, fully automated by computer control software.

Statistical analysis

The data were analyzed by a four-way factorial analysis of covariance (ANCOVA) with peat pellet volume as covariate, using the R software for statistical computing (R-project.org). The categorical factors were tobacco line, P amendment, *G. intraradices* and *P. fluorescens*. Data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Bartlett's test) and log-transformed or arcsine transformed (for percentage data) if necessary. The percentage of mycorrhizal root colonization data was not normally distributed and arcsine transformation unable to correct it. A generalized liner model (GLM) was fitted to the data using quasibinomial error structure to conduct ANOVA-like analysis with F-test.

Results

Covariate

Whenever there was evidence for a correlation between a response variable and the peat pellet volume, the latter was used as covariate within an ANCOVA. The linear model (LM) showed a correlations (P<0.001) between the peat pellet volume and whole plant dry mass, shoot dry mass and root dry mass, but no correlation between the peat pellet volume and the root to shoot ratio. The LM showed also a correlations (P<0.001) between peat pellet volume and all root morphological parameters, i.e. total root length, average root diameter, total root surface area, total root volume and specific root length. Peat pellet volume was also well correlated with the shoot content of carbon (P<0.01), nitrogen (P<0.001) and the shoot carbon to nitrogen ratio (P<0.001).

Mycorrhizal colonization

No mycorrhizal colonization was detected on the non-inoculated control plants or on plants only inoculated with *P. fluorescens*. The addition of *P. fluorescens* increased root colonization (Table 2, P<0.05; Figure 1) and the *35S:CKX2* line had more mycorrhizal colonization compared with the wild type (Table 2, P<0.001; Figure 2). However, the P amendment interacted with the plant line, showing effects with opposite direction on mycorrhization between the tobacco wild type and transgenic *35S:CKX2* line (Table 2, P<0.001; Figure 3). Overall, the mycorrhizal colonization was low. The *35S:CKX2* line growing on low P was colonized the most (9 %) while the wild type growing under the same P amendment was colonized the least (1 %) by *G. intraradices*.

Table 2. ANOVA table from a general linear model (quasibinomial error structure) on the effect of tobacco line (T), P amendment (P), *G. intraradices* (M) and *P. fluorescens* (B) on the percentage of root mycorrhizal colonization.

Source	df	Mycorrhization	
		F	P
T	1	**9,03**	**< 0,001**
P	1	0,45	0,977
M	1	**57,61**	**< 0,001**
B	1	**4,81**	**0,020**
TxP	1	**7,14**	**0,014**
TxM	1	0,00	1,000
PxM	1	0,00	1,000
TxB	1	0,86	0,314
PxB	1	0,67	0,387
MxB	1	0,00	1,000
TxPxM	1	0,00	1,000
TxPxB	1	0,00	0,978
TxMxB	1	0,00	1,000
PxMxB	1	0,00	1,000
TxPxMxB	1	0,00	1,000
Error	112		

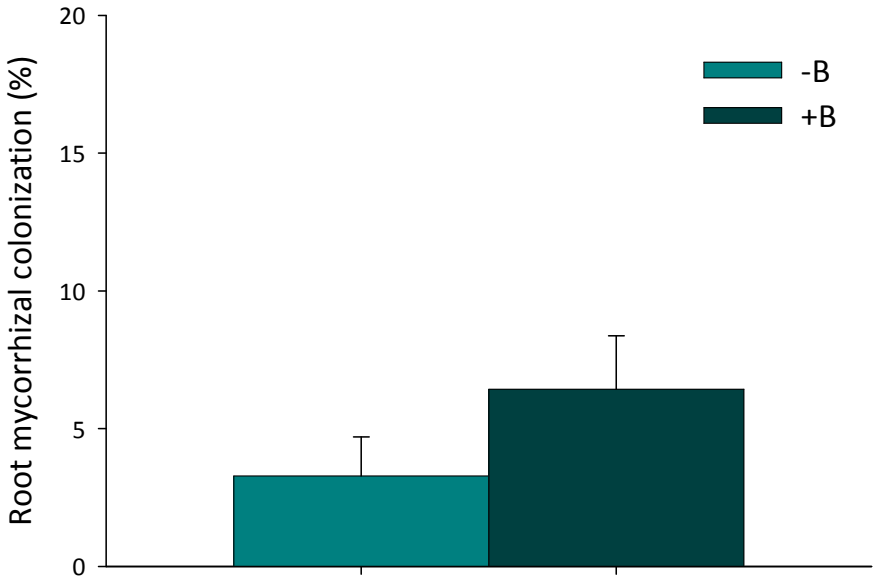

Figure 1. Percentage of root mycorrhizal colonization as affected by *P. fluorescens* presence (+B) or absence (-B). Means +SE. For ANOVA results see Table 2.

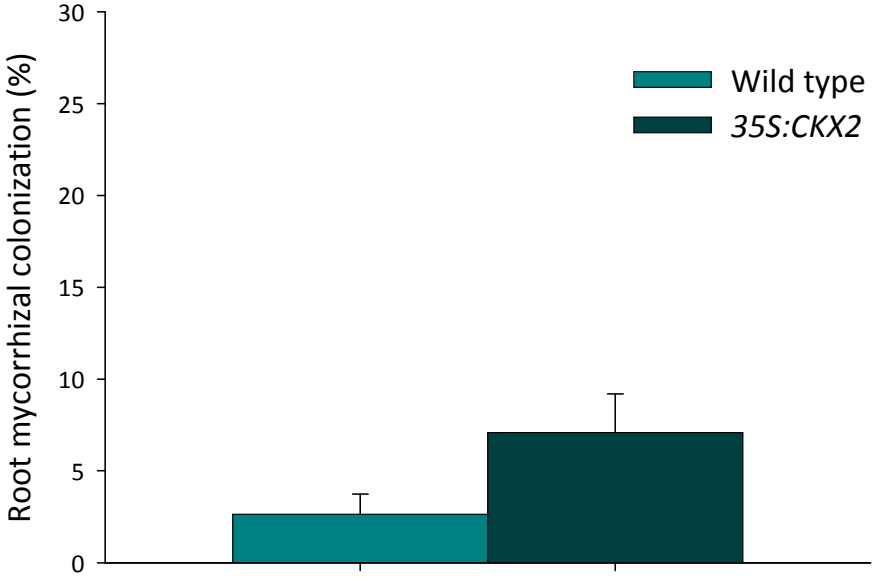

Figure 2. Percentage of mycorrhiza root colonization in the wild type and *35S:CKX2* line. Means +SE. For ANOVA results see Table 2.

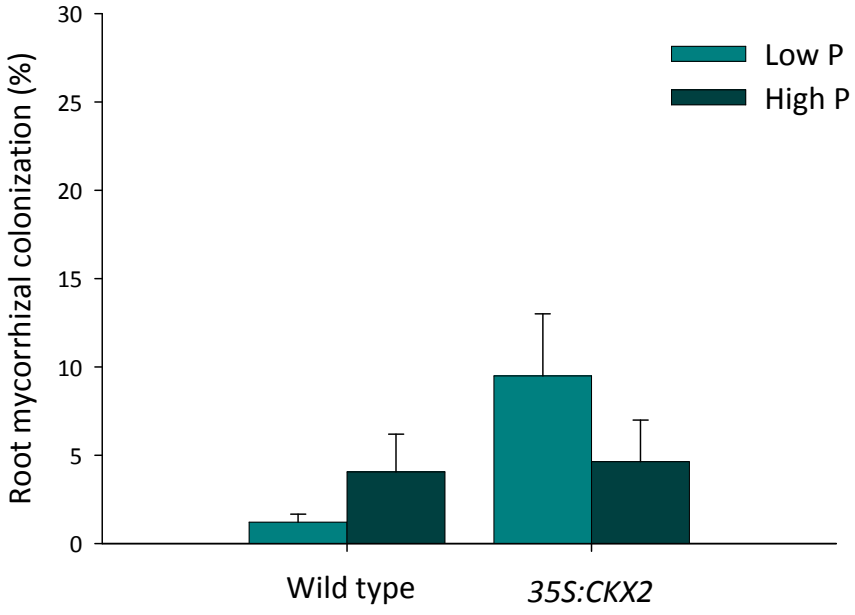

Figure 3. Percentage of mycorrhiza root colonization of the wild type and *35S:CKX2* line as affected by P amendment. Means +SE. For ANOVA results see Table 2.

Plant biomass

A four way interactions including all treatments were detected on whole plant dry mass (Table 3, *P*<0.01) and shoot dry mass (Table 3, *P*<0.01). In Figure 4 it is possible to see that plant dry mass of the wild type growing on low P was negatively affected by single microbial inoculations, with *P. fluorescens* having the strongest negative effect, but not when co-inoculated. On the other hand, the wild type dry mass seems not to be affected by the microbial inoculations, single or in combination, when growing on high P amendment. Furthermore, only in the *35S:CKX2* line growing on low P single inoculation with *P. fluorescens* tended to increase plant dry mass, while the inoculation of *G. intraradices*, alone or in combination with *P. fluorescens*, did not affect plant dry mass compared with the non-inoculated control. Contrastingly, the plant dry mass of *35S:CKX2* growing on high P was negatively affected by single or combined microbial inoculations, with *G. intraradices* having the strongest negative effect. Additionally, the effects observed for the whole plant dry mass are very similar to the ones observed on the shoot dry mass (Figure 5).

Root dry mass was affected by a three way interaction among tobacco line, *G. intraradices* and *P. fluorescens* (Table 3, *P*<0.05). In Figure 6 it is possible to observe that the wild type root dry mass was not much affected by microbial inoculations. Here, only single inoculation of *G. intraradices* seems to negatively affect the root dry mass of the wild type, a trend that disappears when the fungus is co-inoculated with *P. fluorescens*. Contrastingly, the *35S:CKX2* root dry mass is not affected by *G. intraradices* but is positively affected by the *P. fluorescens*, while the co-inoculation results in neutral effects on root dry mass compared with the non-inoculated control.

Tobacco line as a single factor had the strongest significant effect (Table 3, *P*<0.001); the wild type had increased shoot, root and whole plant dry mass. The P amendment as main factor increased whole plant and shoot dry mass on high P (Table 3, *P*<0.05), but had no effects on root dry mass. *G. intraradices* as main factor had significant effects on whole plant, shoot and root dry mass (Table 3, *P*<0.05), while *P. fluorescens* showed significant effects only on root dry mass (Table 3, *P*<0.05). Additional interactions were also detected among P amendment and *G. intraradices* on whole plant, shoot and root dry mass (Table 3, *P*<0.05), and among tobacco line, *G. intraradices* and *P. fluorescens* on whole plant dry mass (Table 3, *P*<0.05). However, the means of these interactions and main factor effects cannot be predicted on additive basis without considering the highest factorial interaction detected.

Table 3. ANCOVA table on the effect of tobacco line (T), P amendment (P), *G. intraradices* (M) and *P. fluorescens* (B) on dry mass (g) of the whole plant, shoot or root and on the root to shoot ratio.

Source	df	Whole plant		Shoot		Root		Root/Shoot	
		F	P	F	P	F	P	F	P
T	1	**282.58**	**< 0.001**	**301.29**	**< 0.001**	**125.42**	**< 0.001**	**26.83**	**< 0.001**
P	1	**5.04**	**0.026**	**6.87**	**0.010**	0.03	0.872	**16.01**	**< 0.001**
M	1	**5.05**	**0.026**	**4.95**	**0.028**	**4.22**	**0.042**	0.13	0.722
B	1	0.59	0.442	0.17	0.685	**5.94**	**0.016**	**13.30**	**< 0.001**
TxP	1	2.21	0.139	2.31	0.131	0.47	0.495	1.01	0.317
TxM	1	0.16	0.689	0.24	0.628	0.03	0.853	0.99	0.322
PxM	1	**9.29**	**0.002**	**10.01**	**0.002**	**4.78**	**0.031**	0.53	0.468
TxB	1	1.90	0.170	2.41	0.123	0.36	0.551	1.41	0.237
PxB	1	0.85	0.358	0.43	0.514	2.71	0.103	3.69	0.057
MxB	1	0.38	0.537	0.57	0.451	0.00	0.969	1.04	0.311
TxPxM	1	0.03	0.866	0.00	0.980	0.64	0.424	1.84	0.177
TxPxB	1	1.77	0.185	2.63	0.107	0.00	0.988	**5.54**	**0.020**
TxMxB	1	**3.96**	**0.048**	3.38	0.069	**5.48**	**0.021**	2.13	0.147
PxMxB	1	0.46	0.501	0.66	0.417	0.01	0.904	0.88	0.349
TxPxMxB	1	**8.64**	**0.003**	**10.29**	**0.002**	1.68	0.198	**5.40**	**0.022**
Error	112								

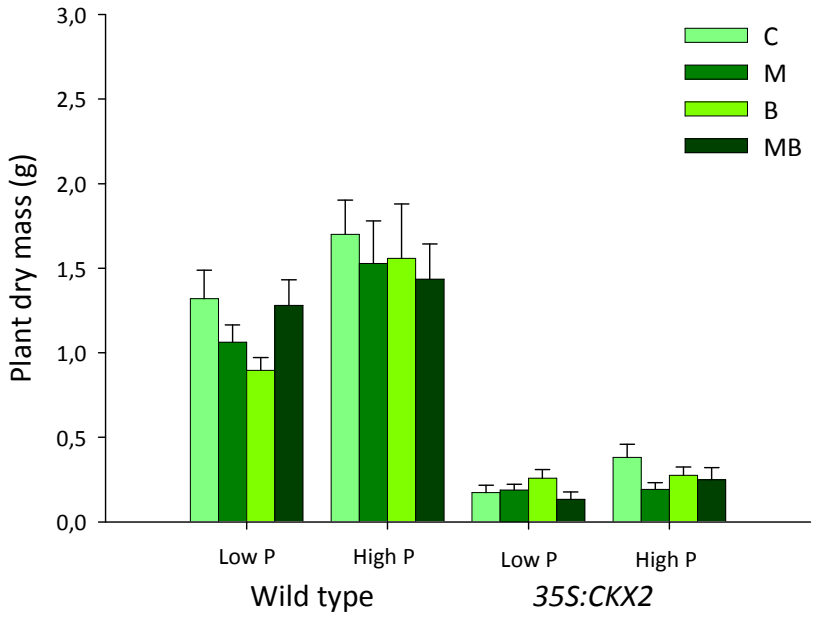

Figure 4. Whole plant dry as affected by tobacco line, P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 3.

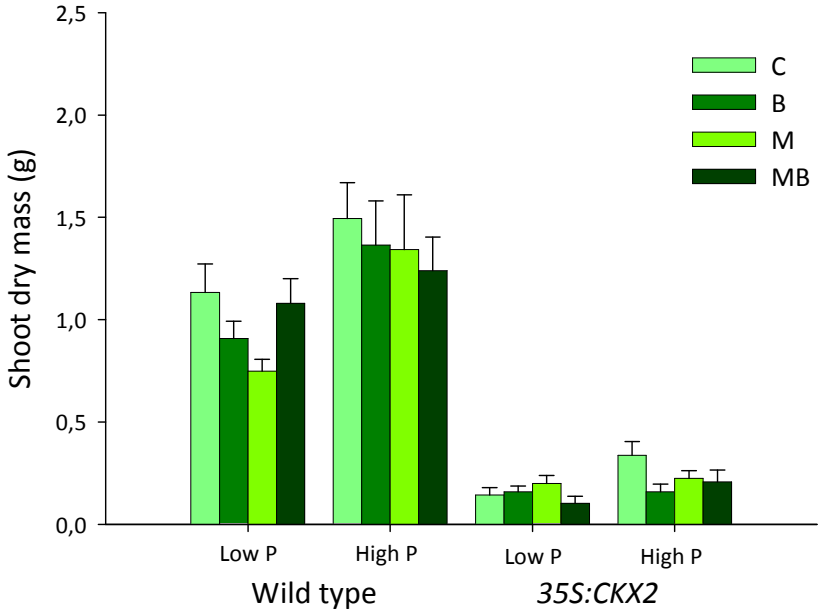

Figure 5. Shoot dry mass as affected by tobacco line, P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 3.

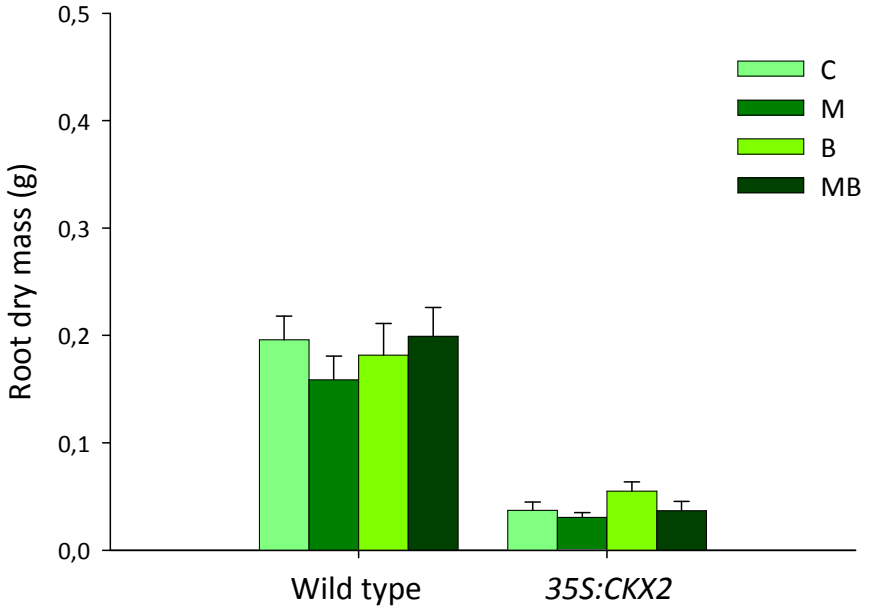

Figure 6. Root dry mass as affected by tobacco line, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 3.

A four way interaction including all treatments was detected on root to shoot ratio (Table 3, $P<0.05$; Figure 7). The wild type growing in low P was increased by *P. fluorescens* alone, slightly increased by the microbial co-inoculation and not affected by single inoculation of *G. intraradices*. On the high P, the wild type root to shoot ratio seems to be slightly decreased by *G. intraradices* alone, while on the other microbial inoculations no obvious changes occurred compared with the respective non-inoculated control. However, on the *35S:CKX2* line growing on low P, *P. fluorescens* alone or in combination with *G. intraradices* enhanced considerably the root to shoot ratio, in comparison to *G. intraradices* alone or to the non-inoculated control. On high P, the *35S:CKX2* root to shoot was positively affected by all microbial inoculations, with the strongest effect of *G. intraradices*, followed by *P. fluorescens* and the co-inoculation.

Tobacco line, P amendment and *P. fluorescens* as main factors had significant effect on the root to shoot ration (Table 3, $P<0.001$). Furthermore, a three way interactions including tobacco line, P amendment and *P. fluorescens* were also detected (Table 3, $P<0.05$).

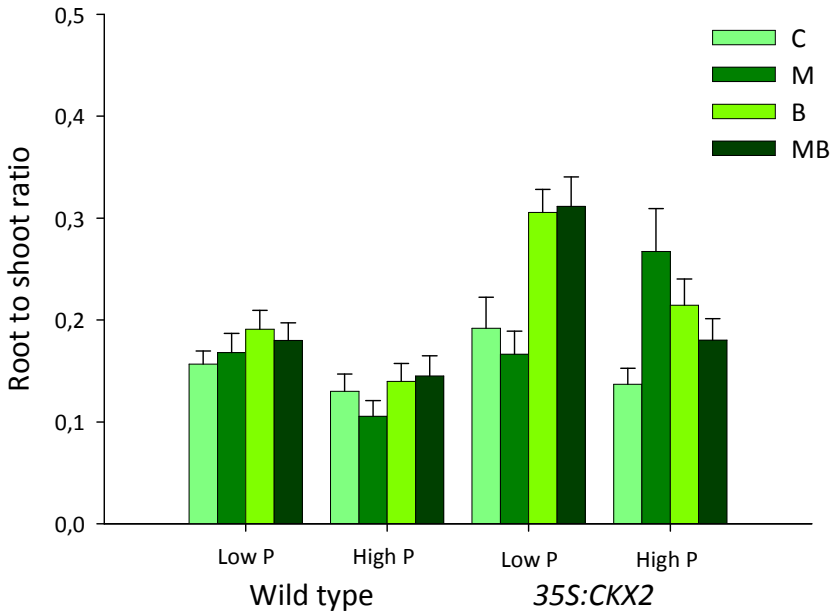

Figure 7. Root to shoot dry mass ratio as affected by tobacco line, P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 3.

Root morphology

A four way interactions including all treatments were detected on total root length (Table 4, $P<0.05$; Figure 8). The wild type growing on low P was negatively affected by *P. fluorescens*, positively affected by co-inoculation and not affected by *G. intraradices* alone. The wild type growing in high P was not apparently affected by any inoculation. The *35S:CKX2* line growing on low P seems to have a slight increase in total root length when inoculated with *P. fluorescens*, which is substantially higher than the co-inoculation or *G. intraradices* alone. When the *35S:CKX2* line was grown on high P, the *G. intraradices* reduced the total root length compared with the non-inoculated control, but no changes were observed for the other inoculations.

The root average diameter had also a four way interactions (Table 4, $P<0.05$; Figure 10). The root diameter in wild type grown on low P was decreased by *G. intraradices* alone; no major effects were observed of the *P. fluorescens* single inoculation or the microbial co-inoculation compared with the non-inoculated control. On high P, the wild type does not seem to be affected by the microbial inoculations. The *35S:CKX2* root average diameter on low P tended to be positively affected by both single inoculations but was not affected by co-inoculation. On high P, the microbial inoculations did not affect much the root average diameter of the *35S:CKX2* line, however, among the single inoculations, *P. fluorescens* tended to induce higher root diameters compared with *G. intraradices*.

A four way interactions were detected on the total root surface area and volume (Table 4, $P<0.05$). The total root surface area (Figure 11) of the wild type growing on low P is decreased by *P. fluorescens* and increased by co-inoculation, while *G. intraradices* had no effect compared with the non-inoculated control. On high P, the microbial inoculations did not influence substantially the total root surface area of the wild type. On the *35S:CKX2* line growing on low P, no major changes occurred, except of the single inoculation of *P. fluorescens*, which increased root surface area compared with the co-inoculation and showed a trend for increased root surface area compared with the non-inoculated control. On high P, the *35S:CKX2* was negatively affected by *G. intraradices*, but not by *P. fluorescens* or the co-inoculation, compared to the non-inoculated control. Furthermore, the effects on total root surface area and on total root volume, caused by the microbial inoculations, have similar patterns (Figure 12).

Table 4. ANCOVA table on the effect of tobacco line (T), P amendment (P), *G. intraradices* (M) and *P. fluorescens* (B) on total root length (cm), average root diameter (mm), total root surface area (cm^2), total root volume (cm^3) and specific root length ($cm\ g^{-1}$).

Source	df	Length		Average diameter		Surface area		Volume		Specific length	
		F	P	F	P	F	P	F	P	F	P
T	1	**23,59**	**< 0,001**	**14,46**	**< 0,001**	**23,70**	**< 0,001**	**23,70**	**< 0,001**	**33,68**	**< 0,001**
P	1	0,58	0,448	**5,91**	**0,017**	0,04	0,833	0,04	0,803	**20,91**	**< 0,001**
M	1	1,81	0,182	0,41	0,524	1,53	0,218	1,53	0,260	3,16	0,078
B	1	1,34	0,250	3,56	0,062	1,94	0,166	1,94	0,122	**7,41**	**0,008**
TxP	1	1,29	0,258	1,82	0,180	1,73	0,191	1,73	0,150	0,36	0,550
TxM	1	2,86	0,094	0,25	0,620	2,42	0,123	2,42	0,154	3,72	0,056
PxM	1	**4,01**	**0,048**	1,00	0,319	3,10	0,081	3,10	0,130	0,09	0,764
TxB	1	0,00	0,994	0,12	0,730	0,03	0,855	0,03	0,738	1,32	0,252
PxB	1	0,85	0,359	2,55	0,113	1,43	0,234	1,43	0,160	3,11	0,081
MxB	1	1,30	0,256	0,49	0,485	1,22	0,272	1,22	0,294	0,07	0,797
TxPxM	1	1,84	0,178	0,64	0,424	1,47	0,228	1,47	0,281	0,98	0,324
TxPxB	1	1,29	0,259	3,88	0,051	1,92	0,168	1,92	0,116	0,29	0,594
TxMxB	1	1,41	0,238	**5,25**	**0,024**	1,86	0,176	1,86	0,142	**8,80**	**0,004**
PxMxB	1	0,08	0,776	0,02	0,889	0,26	0,612	0,26	0,494	**4,57**	**0,035**
TxPxMxB	1	**4,43**	**0,038**	**7,50**	**0,007**	**5,06**	**0,026**	**5,06**	**0,021**	**7,20**	**0,008**
Error	112										

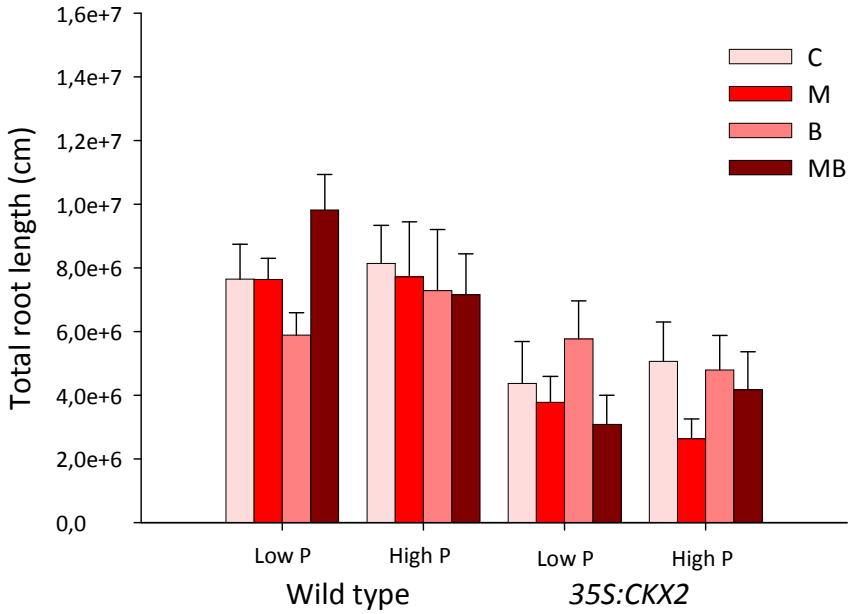

Figure 8. Total root length as affected by tobacco line, P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 4.

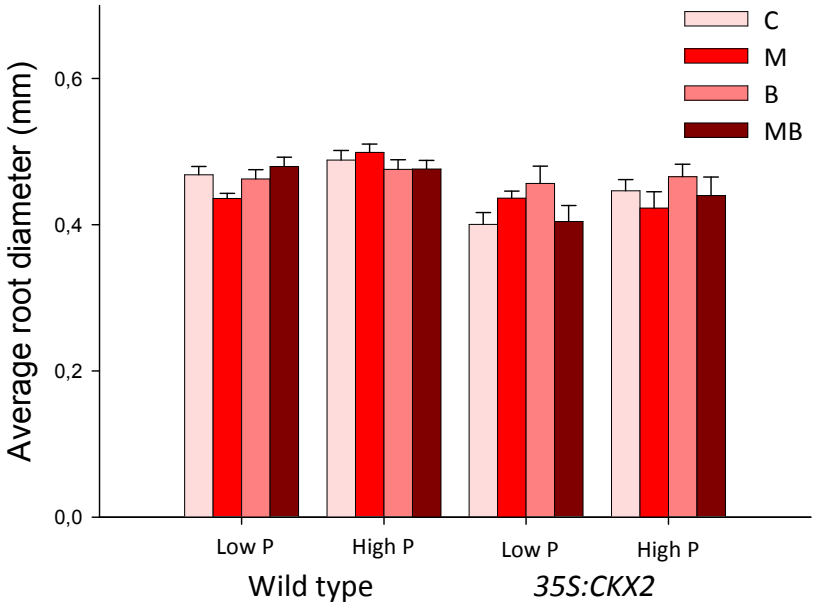

Figure 9. Average root diameter as affected by tobacco line, P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 4.

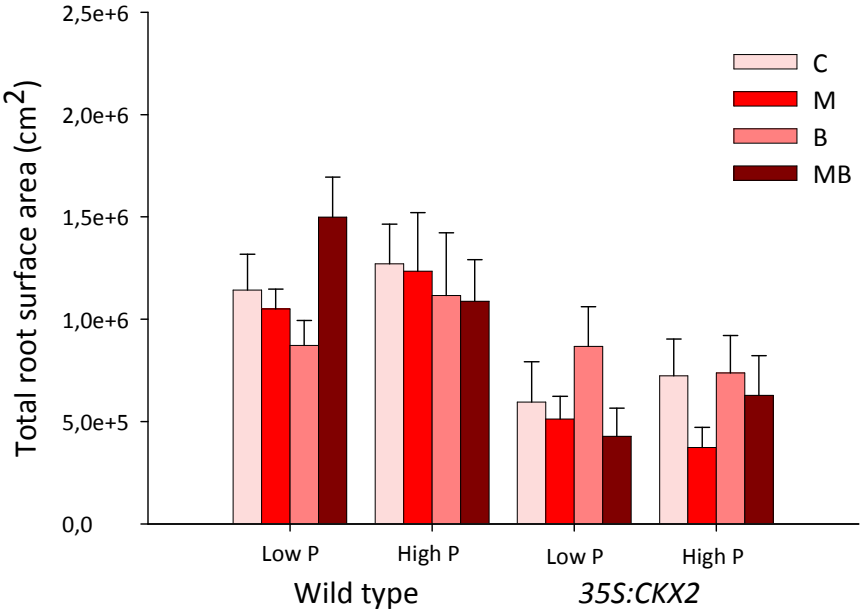

Figure 10. Total root surface area as affected by tobacco line, P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 4.

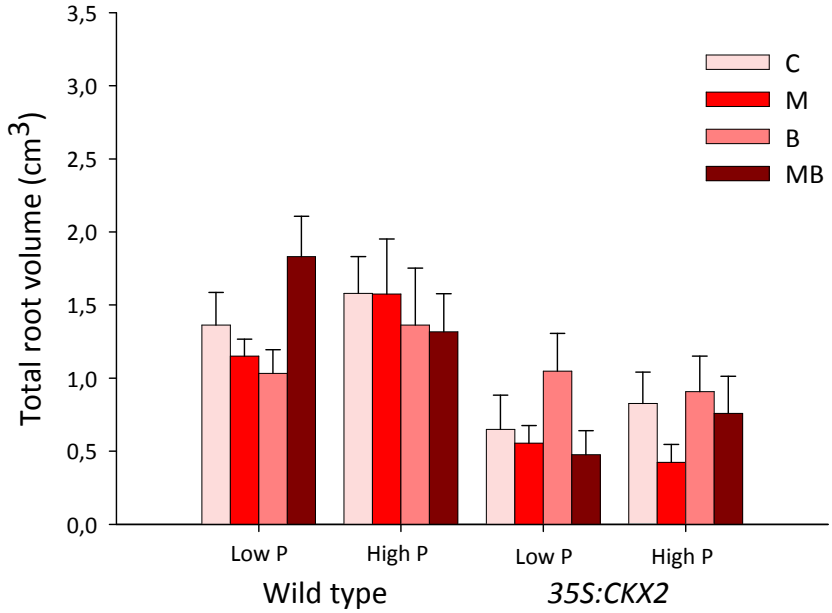

Figure 11. Total root volume as affected by tobacco line, P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 4.

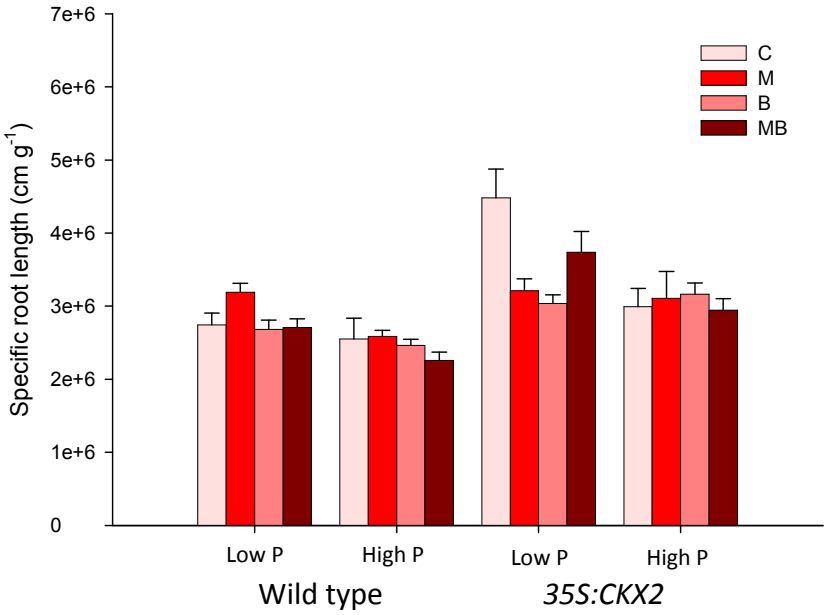

Figure 12. Specific root length as affected by tobacco line, P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 4.

A four way interactions including all treatments were detected on specific root length (Table 4, $P<0.01$; Figure 12). The wild type growing on low P was positively affected by *G. intraradices*, but not affected by *P. fluorescens* or the co-inoculation. The wild type growing in high P was not substantially affected by any inoculation, only co-inoculation shows a trend to a slight decrease. The specific root length of the *35S:CKX2* line growing on low P was reduced by all microbial inoculations, with the co-inoculation having a less negative effect. When the *35S:CKX2* line was grown on high P, the specific root length was not much affected by any microbial inoculation.

Tobacco line as main factor had significant effect on the five root morphological parameters (Table 4, $P<0.001$). P amendment as main factor had significant effect on the average root diameter and specific root length (Table 4, $P<0.05$). *P. fluorescens* as main factor had effect on the specific root length (Table 4, $P<0.01$). In addition, a two way interaction was detected among between P amendment and *G. intraradices* on total root length (Table 4, $P<0.05$), a three way interactions among tobacco line, *G. intraradices* and *P. fluorescens* on the average root diameter and specific root length (Table 4, $P<0.05$ and $P<0.01$, respectively), and a three way interactions among P amendment, *G. intraradices* and *P. fluorescens* on the specific root length (Table 4, $P<0.05$).

Carbon and nitrogen content in the shoot

A four way interaction including all treatments was detected on carbon content in the shoots (Table 5, $P<0.05$; Figure 13). The wild type carbon content on low P was slightly increased by the co-inoculation compared with the non-inoculated control. On high P, there is a trend to decreased carbon content in the wild type due the microbial inoculations. The *35S:CKX2* line shows a different patter. On low P, all microbial inoculation resulted in decreased carbon content, with single *G. intraradices* inoculated plants having the lowest value. Contrastingly, on high P, *G. intraradices* inoculation alone resulted in increased carbon content compared to the non inoculated control, while inoculation with *P. fluorescens* or the co-inoculation only slightly increased the carbon content.

The nitrogen content showed a four way interaction (Table 5, $P<0.05$; Figure 14). The wild type on low P had a slight trend for increased nitrogen content due to the microbial inoculations. On high P, nitrogen content in the wild type has a tendency to increase when inoculated with *P. fluorescens*, while the other inoculations did not substantially affected the nitrogen content. The co-inoculation showed a reduction on nitrogen content compared with *P. fluorescens* single inoculation. Furthermore, the *35S:CKX2* nitrogen content on low P is positively affected by single inoculations but not affected by co-inoculation compared with the non-inoculated control. On high P, all microbial inoculations decreased the nitrogen content, with the effects being slightly stronger in the single inoculations.

A three way interactions were detected for the carbon to nitrogen ratio (Table 5, $P<0.05$; Figure 15). When the plants were grown on low P, all microbial inoculations had a negative effect on the C/N ratio in comparison to the non-inoculated controls, while on high P, no major changes were observed.

Tobacco line and *G. intraradices* as main factors had significant effects (Table 5, $P<0.001$ and $P<0.05$ respectively) on the carbon and nitrogen content as well as on carbon to nitrogen ratio. Two way interactions were detected among *G. intraradices* and *P. fluorescens* on carbon to nitrogen ratio (Table 5, $P<0.05$) and among tobacco line and P amendment on carbon content (Table 5, $P<0.001$). Three way interactions were detected among tobacco line, P amendment and *G. intraradices* on carbon content (Table 5, $P<0.05$) and among tobacco line, *G. intraradices* and *P. fluorescens* on carbon and nitrogen content (Table 5, $P<0.05$).

Table 5. ANCOVA table on the effect of tobacco line (T), P amendment (P), *G. intraradices* (M) and *P. fluorescens* (B) on the shoot content of carbon and nitrogen (%) and on carbon to nitrogen ration.

Source	df	Carbon		Nitrogen		Carbon/Nitrogen	
		F	P	F	P	F	P
T	1	**382,55**	**< 0,001**	**128,10**	**< 0,001**	**185,02**	**< 0,001**
P	1	1,92	0,169	0,76	0,386	0,58	0,447
M	1	**5,77**	**0,018**	**4,44**	**0,037**	**4,33**	**0,040**
B	1	0,11	0,736	0,56	0,454	0,09	0,767
TxP	1	**10,39**	**0,002**	1,97	0,163	3,81	0,053
TxM	1	0,23	0,634	0,74	0,392	1,11	0,294
PxM	1	0,29	0,589	0,01	0,913	0,10	0,751
TxB	1	0,92	0,341	0,00	0,994	0,00	0,983
PxB	1	0,48	0,490	0,09	0,760	0,08	0,784
MxB	1	2,68	0,104	3,78	0,054	**4,75**	**0,031**
TxPxM	1	**7,83**	**0,006**	0,02	0,886	0,02	0,882
TxPxB	1	1,95	0,165	0,56	0,455	0,31	0,580
TxMxB	1	0,21	0,647	0,32	0,575	0,64	0,427
PxMxB	1	**5,95**	**0,016**	**4,82**	**0,030**	**5,56**	**0,020**
TxPxMxB	1	**5,64**	**0,019**	**5,54**	**0,020**	2,30	0,132
Error	112						

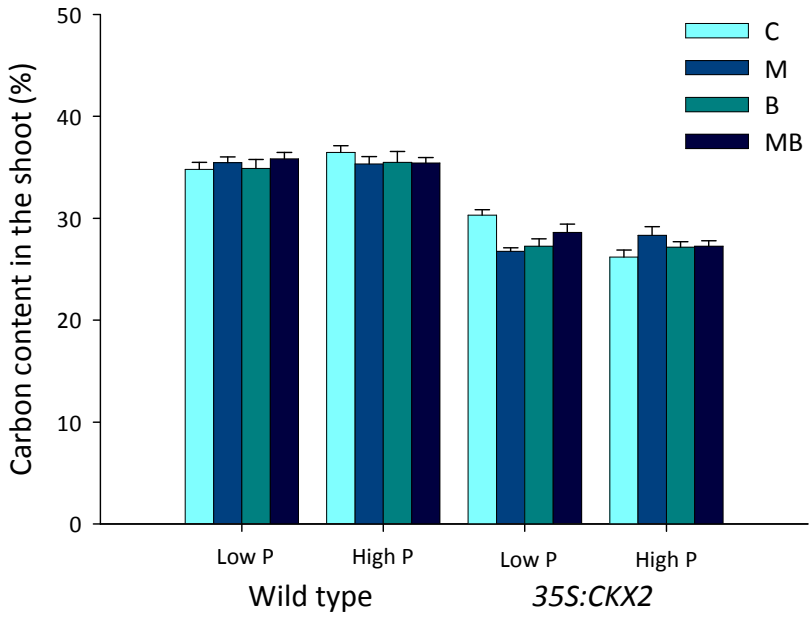

Figure 13. Carbon content in the shoot as affected by tobacco line, P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 5.

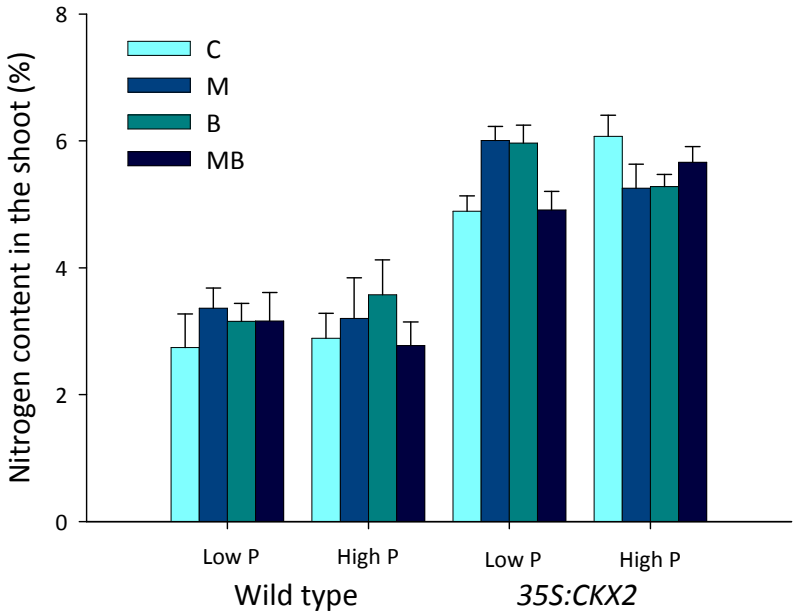

Figure 14. Nitrogen content in shoot as affected by tobacco line, P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 5.

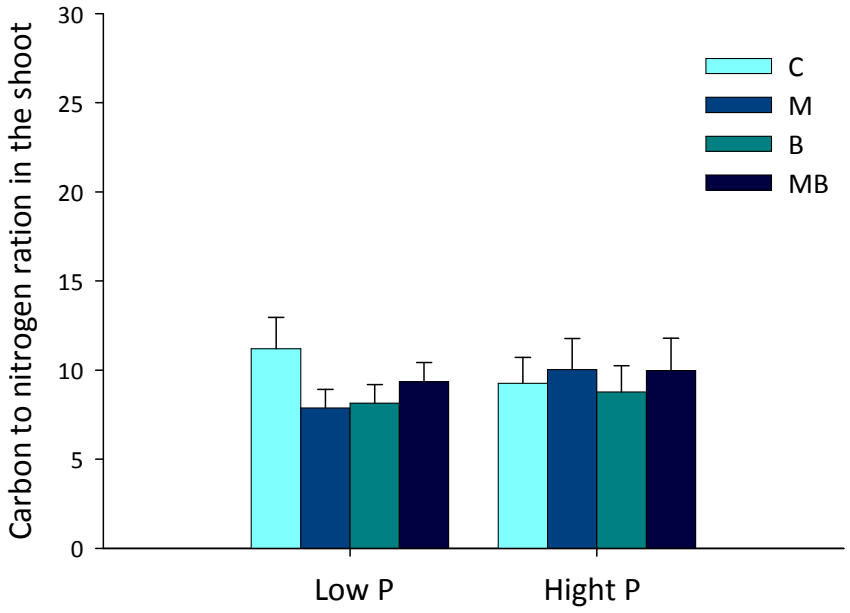

Figure 15. Carbon to nitrogen ratio in the shoot as affected by P amendment, inoculation of *G. intraradices* (M) and *P. fluorescens* (B), alone or in combination (MB), and the non-inoculated control (C). Means +SE. For ANCOVA results see Table 5.

Discussion

Comparing non-inoculated controls among plant lines and P amendments

The tobacco lines used in this study have been previously described (Werner *et al.* 2001; Werner *et al.* 2008); *35S:CKX2* is a cytokinin deficient line and develops slow-growing, stunted shoots with small leaves and an enhanced root system compared with its wild type.

In the present study, the wild type non-inoculated control growing on low P had reduced plant dry mass compared with the non-inoculated control on high P. No major changes occurred in the root morphology, but the root to shoot ratio was slightly reduced on high P. It is widely recognized that deficiencies of N and P result in higher levels of carbon allocated to the root and an increased root to shoot biomass ratio (Hermans *et al.* 2006). The wild type non-inoculated controls had the same N content in the shoot, but on low P the C content was decreased. P is critical for photosynthesis, plant growth and metabolism because P is a key ingredient in cell energetics through adenosine triphosphate (ATP) production, and also plays a key role in protein synthesis as a component of nucleic acids and lipid membranes (Johnson 2010). The results suggest that root to shoot ratio didn't play a significant role in N uptake, and that P was comparatively limited in the wild type growing on low P, with negative effects on the shoot content in photosynthetically fixed C. However, the differences in P availability were not enough to cause substantial root morphological changes, as generally reported (Hermans *et al.* 2006).

A similar situation occurred between the *35S:CKX2* growing on low and high P; the latter had increased plant dry mass, reduced root to shoot ratio, and no changes occurred on the total root length, surface area and volume. However, the average root diameter was clearly enhanced on high P and the specific root length increased on low P. These results suggest that P was also comparatively limited on low P amendment, but when the plant is deficient in cytokinin, changes occurred in the root thickness depending on P availability.

In roots, auxin is involved in lateral root formation, maintenance of apical dominance and adventitious root formation (Hodge *et al.* 2009). CK promotes cell differentiation by repressing both auxin transport and responses to auxin at the boundary between the meristem and the root elongation zone (Chapman & Estelle 2009), as a result CK suppresses lateral root formation, but its reduction

display increased root branching and enhanced primary root growth (Werner *et al.* 2003). At the whole plant level, two types of response are activated when P is deficient. The first depends on external ion concentration and involves local signals. The second depends on whole plant mineral status and involves long-distance signaling (Hermans *et al.* 2006). When plants are P deficient, root growth accelerates and augmented lateral root branching further increases the foraging capacity of the root system, which seem to be associated with local auxin signaling (Hermans *et al.* 2006; Hodge *et al.* 2009). Long-distance signals mediating the shoot response to P perception in roots seem to involve CK. It is possible that the reduction in CK observed during P starvation (Franco-Zorrilla *et al.* 2005) relieves a general inhibition of root growth by this hormone (Werner *et al.* 2003), and that an increase in auxin stimulates lateral root development, explaining the higher P sensitivity of specific root length and/or average root diameter on the *35S:CKX2*.

The N content in the shoot of *35S:CKX2* was higher on the high P, possibly due to a thicker root system which generally favors the water translocation as well as the uptake of NO_3^- (Richardson *et al.* 2009), the molecular form of N supplied in the present experiment with the nutrient solution.

Comparing microbial effects within each plant line and P amendment

Wild type on low P

The plant dry mass of the wild type growing on low P was negatively affected by *G. intraradices* alone compared with the non-inoculated control. AMF reduction of plant growth has been previously reported and the relation considered parasitic (i.e. the AM symbiosis net cost exceed the net benefits to the plant, reducing plant growth) (Johnson *et al.* 1997). However, *G. intraradices* itself did not perform as expected. In this treatment, AMF percentage of root colonization was very low (approximately 2%) compared with the previously reported (approximately 80% and 60%, respectively) by *Maier et al.* (2000) and Medina *et al.* (2003), using the same tobacco wild type and fungal species and similar periods of plant growth. *G. intraradices* is recognized as one of the most rapid and extensive root colonizer among AMF, and its colonization can be detected early in the first week and reach approximately 80% (Hart & Reader 2002). This suggests that a mutually detrimental relation occurred between *G. intraradices* and the tobacco wild type.

Wurst *et al.* (2004), while investigating the effects of earthworms and *G. intraradices* on the growth and chemistry of *Plantago lanceolata*, found that although shoot biomass was not affected by the fungus, the mycorrhizal plants were more depleted in N than the non-mycorrhizal plants, suggesting that *G. intraradices* may have been competing with the roots for N. Competition between plants and soil microbes has been considered one of the most critical factors affecting the ability of plants to acquire N from soil (Kaye & Hart 1997). However, Inselsbacher *et al.* (2010) studied the effect of different inorganic nitrogen fertilizer on the competition between plants and soil microbes, including fungi, and found that soil microbes out competed plants in the first 4 hours following fertilization, but within one day microbial N uptake declined substantially, probably due to carbon limitation. After a week about 45 to 80% of initially applied ^{15}N was recovered in crop plants compared to only 1 to 10% in soil microbes, showing that plants were the strongest competitors for inorganic N.

In the present work, the N content in the shoot of the wild type on low P inoculated by *G. intraradices* was not decreased; on the contrary, it shows a slightly trend to increase compared with the non-inoculated control. Actually, AMF have been suggested to directly contribute to acquisition of N (He *et al.* 2003). Nevertheless, when considering the role AMF play in soil-to-plant N transfer, attention should be paid to the quantitative contribution to this pathway, bearing in mind that generally plants require about 10 times more N than P (Smith & Read 2008). Additionally, the mechanism by which AMF

transfer N to the host rely much on amino acids formation (Govindarajulu *et al.* 2005). The inorganic N is taken up by the fungus outside the roots and is incorporated into amino acids, then is translocated from the extraradical to the intraradical mycelium as arginine, and transferred to the plant without carbon (Govindarajulu *et al.* 2005). This implies that the AMF contribution to N uptake depends on C availability to the fungus for amino acid formation. Because *G. intraradices* is a poor soil colonizer and a strong root colonizer (Hart & Reader 2002), the low percentage of root colonization detected (2%) shows a poor fugal growth and evidences an extreme limitation of C supply to the fungi. In the light of this fact, it is very unlikely that the fungus was able to play any significant role in the direct soil-to-plant N transfer. However, despite the low percentage of root colonization, *G. intraradices* was able to substantially alter root morphology of the wild type, by enhancing the specific root length and reduce the average root diameter. AMF colonization influences root architecture of the host plant in most studies by causing a more profusely branched root system (Price et al. 1989; Yanoet al. 1996; Paszkowski et al. 2002; Olah et al. 2005; Gutjahr et al. 2009). In *Medicago truncatula*, perception of a diffusible signal released by G. *intraradices* was sufficient to induce lateral root formation before colonization toke place; however, the cellular target for the stimulation is unknown (Olah *et al.* 2005). This suggests that the low percentage of established colonization in the wild type on low P may have been enough to induce the root morphological changes. The increased root specific length seems to explain the shoot N content, when analyzing both parameters compared to those in the other microbial inoculations and control. Enhanced specific root length is generally seen as a root foraging response associated with the uptake of immobile nutrients such as P rather than mobile nutrients such as N (Fitter *et al.* 2002). According to the theoretical model, a profusely branched root system should be as effective to capture the highly mobile N as a more restricted root system, base on the idea that mass flow would be enough for N migration and up take. However, this is not always the case, and despite N mobility, plants can generally develop a profusely branched root system when N is deficient (Hermans *et al.* 2006), and in particular when faced with competition by neighboring plants in order to secure N uptake (Hodge 2004). The results suggest that competition between AMF and plants may have played also a role in the enhancement of the specific root length.

The plant dry mass of the wild type growing on low P was also negatively affected by *P. fluorescens* alone compared with the non-inoculated control. The strain of *P. fluorescens* used here was previously reported as a PGPR by antagonism against root pathogenic fungi and the depression of plant growth was unexpected. However, it is known that soil Pseudomonas can be inhibitory, neutral or positive to plant

growth depending on bacteria species, density and environment (Watt *et al.* 2003). Additionally, it has been also reported that the effect of individual isolates of Pseudomonas on plants can fluctuate from growth inhibition to growth promotion, according to environmental conditions, the host genotype and mycorrhizal status (Nehl *et al.* 1997). Several mechanisms for growth inhibition have been suggested, and include phytotoxins production, destruction of root hairs and production of phytohormones such as auxin, which inhibit root elongation and decreases root to shoot ratio (Loper & Schroth 1986). In the present treatment, *P. fluorescens* did not decrease the root to shoot ratio; on the contrary, it slightly increased. However, it drastically decreased the total root length without affecting the average root diameter and, consequently, decreased the total root surface area and volume. These root morphological changes did not affect the N content in the shoot, suggesting that other factor rather than N limitation was responsible for the plant growth inhibition. P may have been limited for the plant growth due to the reduced root morphological parameters. Additionally, the inhibitory function of *P. fluorescens* on plant dry mass could also be associated with the high bacteria density added into the soil. It is known that bacteria density in the soil is a factor that influences the detrimental effects of Pseudomonas. Bolton & Elliott (1989) showed that toxin production was constitutive and therefore the quantity of toxin was associated with proliferation of the bacterium. Alström (1987) found that a *Pseudomonas* isolate caused disease when the inoculum contained 6×10^{10} cfu ml^{-1}, but lower concentrations of the same isolate caused stunting but not disease. Alström (1987) added a range of Pseudomonas concentration from $6x10^{11}$ to $6x10^{8}$ CFUs per plant growing in 1L pot. In the present study each seedling received $6x10^{9}$ into a 500 ml container, which is a high CFUs concentration. Watt *et al.* (2003) reported that an accumulation around the root tip of wheat was particularly important for the inhibitory activity of Pseudomonas, and that this inhibition was negatively correlated with the rate of root elongation. Faster rates of root elongation decreased the Pseudomonas accumulation around the root tip by spreading its colonization along the root elongation zone. They suggested also that the root meristems is more sensitive than other zones of the root to compounds produced by Pseudomonas. Although in the present study it was not possible to determine the root colonization of *P. fluorescens*, and access a correlation between its density and the plant growth inhibition, one possible picture can be drawn between its inhibitory function and the total root length along the different treatments, whether *G. intraradices* was present or not and independently on P amendment. For example, when the tobacco wild type on low P was co-inoculated by both microorganisms the total root length was considerably enhanced and the inhibitory effect of *P. fluorescens* disappeared. In *35S:CKX2* on low P inoculated with *P. fluorescens* alone showed a tendency to increased total root length, and this is associated also with a

tendency to enhanced plant dry mass. However, when the same tobacco line on low P was co-inoculated, the total root length tends to be reduced as well as the plant dry mass. On high P, the tobacco wild type root length was not much affect by *P. fluorescens* neither was its plant dry mass. This suggests that when the *P. fluorescens* inoculated plants had a reduced root length compared with the controls the plant growth was inhibited, and when they had increased root elongation the plant growth was enhanced.

The plant dry mass of the wild type growing on low P was not affected by co-inoculation with *G. intraradices* and *P. fluorescens* compared with the non-inoculated control. However, substantial changes were detected on the root morphology, with the most evident effect being the enhanced total root length. No changes occurred on the average root diameter or on the specific root length. As result, total root surface area and volume was enhanced mostly due to an increase root length. These enhanced morphological parameters did not result in enhanced N uptake, suggesting that the presence of both microorganisms obligate the plant to have a more developed root system to maintain the same N uptake; sustaining the previously hypothesized plant-microbe competition for N. Additionally, the AMF colonization of the wild type on low P increased from 2% in the *G. intraradices* single inoculation to 3% in the co-inoculation. The inhibitory effect that *G. intraradices* had alone on plant dry mass and that does not occur in the co-inoculation, is not accompanied by a reduction of mycorrhizal colonization, suggesting that competition for N and changes in root morphology were more relevant than fungal C demand.

The increased root length, which can be translated in faster root elongation, may have been the reason why *P. fluorescens* inhibitory function disappeared in the co-inoculated wild type on low P, as suggested above and explained by Watt *et al.* (2003). In the wild type on low P the combination of both microorganisms seems to be accumulative. Gamalero *et al.* (2004) also found acumulative effects of *G. mosseae* and *P. fluorescens* on tomato root architecture. While *G. mosseae* alone enhanced total root surface area and volume, number of tips and degree of root branching, the co-inoculation with *G. mossae* and *P. fluorescens* 92rk increased all parameters, and enhanced in particular the total root length. In the present study, the positive effects on root morphology were not accompanied by increase in plant dry mass compared with the non-inoculated control, but were enough to cancel the inhibitory effect of both microorganisms isolates.

Wild type on high P

The microbial inoculations, alone or in combination, did not affect the plant dry mass of the wild type on high P. Total root length, average root diameter, total root surface area and volume and the specific root length were not affected as well. Only the microbial co-inoculation slightly reduced the specific root length in comparison with *G. intraradices* or *P. fluorescens* alone. When comparing with low P, the mycorrhizal colonization was higher (8 and 9% on *G. intraradices* alone or co-inoculated with *P. fluorescens*, respectively), showing that the high P availability in the soil did not result in depressed mycorrhization; on the contrary, it sustained its increase. This reinforces the previous proposed hypothesis that the C demand from the fungi was not a major factor inhibiting plant dry mass, as observed on low P. The changes in the root morphology parameters, in particular the enhanced average root diameter and reduction of specific root length seems to explain why *G. intraradices* loses its inhibitory previously observed on low P. The mechanism for this root morphological change is difficult to explain, but is possible that involves better plant fitness due to high P availability, allowing the root to adapt more actively to the soil biota during early foraging activity. *P. fluorescens* has also no effects on plant dry mass or root morphology; supporting the previously hypothesized that *P. fluorescens* inhibitory function is closely associated with the root elongation.

These results suggest that both microorganism effects on plant dry mass depended on P amendment and that these effects were associated with changes in the root morphology. When *G. intraradices* reduced averaged root diameter or *P. fluorescens* reduced the total root length, the outcome plant dry mass was reduced. Gamalero *et al.* (2002) suggested that *G. mosseae* or *P. fluorescens* effects on tomato root morphology, and consequently on plant growth, were dependent on the soil nutrient availability. In the present study, the effects of the co-inoculation with *G. intraradices* and *P. fluorescens* on root morphology also depended on P availability; however, despite of the changes in root morphology, the effects of the co-inoculation of the microorganisms on plant dry mass were not dependent on P. Nevertheless, this seems to be a merely context dependent situation. When considering the previously reported (Gamalero *et al.* 2002; Gamalero *et al.* 2004) and the results presented in this study, one can conclude that the positive microorganism effects on root morphology only resulted in enhanced plant growth if the positive effects on root morphological parameters were strong enough to improve acquisition of nutrients and avoidance of microorganisms deleterious effects (e.g. competition for nutriments, phytotoxins production or plant C sequestration).

35S:CKX2 on low P

The plant dry mass of the *35S:CKX2* growing on low P was not affected by *G. intraradices* alone compared with the non-inoculated control, neither was the total root length, surface area and volume. However, the average root diameter was enhanced and, consequently, the specific root length was reduced. The N content in the shoot seems to benefit with the increase in average root diameter, in conformity with the theory that thicker roots are favorable to greater water transport and N uptake (Richardson *et al.* 2009). When comparing the root morphology of the *35S:CKX2* with the wild type growing on low P, it seems that the thicker root system was more important for N uptake than the enhanced specific root length. Additionally, mycorrhizal colonization was kept on check (10%) in what seems to be a commensalism relation (Johnson 2010). The plant growth was not affected by mycorrhizal colonization, but the fungus performed better than observed in the wild type, although it did not colonize the root with the extent generally reported (Maier *et al.* 1995; Hart & Reader 2002; Medina *et al.* 2003). A commensalism relation, as described by Johnson *et al.* (2010), is predicted when P is not limited, so plants have nothing to gain from C-for-P trade, but C demand by AM fungi is kept in check, generally because fungal growth is N-limited. When comparing the present treatment with the wild type on low P, the mutual detrimental relation between *G. intraradices* and plant disappeared, the N content in the shoot as well as the mycorrhization were increased, and this is associated with reduced specific root length. These results suggest that the plant is not limited by N uptake, and support the previously suggested that enhancement of the specific root length may have been a result from competition between the AMF and plant roots.

The plant dry mass of the *35S:CKX2* growing on low P was slightly enhanced by *P. fluorescens* alone compared with the non-inoculated control. Total root length, surface area and volume tended to be enhanced, while the average root diameter was clearly increased, and consequently the specific root length was reduced. Furthermore, the root to shoot ratio was drastically enhanced, showing that the increased plant dry mass was accompanied by a strong root growth. The trend to enhanced total root length is in conformity with the hypothesis that root elongation reduces the inhibitory function of Pseudomonas (Watt *et al.* 2003). Plant growth promotion by *P. fluorescens* has been ascribed to the suppression of phytopathogenic soilborne microorganisms and to direct effects on plant physiology (Lugtenberg & Kamilova 2009). The increased root morphological parameters accompanied by increased root to shoot ratio were associated with enhanced N content in the shoot to similar levels as observed in *G. intraradices* alone. These results suggest that the plant dry mass increase was not only associated

with increased N uptake and that other factors may have played an important role. For example, enhanced total root surface and volume may have contributed to increased P uptake. Gamalero *et al.* (2004) reported that *P. fluorescens* enhanced total root length, surface area and volume on tomato plants, leading to increased leaf P content and whole plant biomass. Whenever *P. fluorescens* was present, the root to shoot ratio tended to be enhanced independently of on the effect on plant dry mass (i.e. if negative or positive), and this effect was negatively related with P availability or plant CK content. For example, the root to shoot ratio of the *35S:CKX2* growing on low P was drastically enhanced by *P. fluorescens*, independently of *G. intraradices* presence. The auxin synthesis *by P. fluorescens* has been related to the enhancement of root system (Lugtenberg & Kamilova 2009). In the present study, the strain used was not tested for its ability to synthesize auxin, neither there was reference that could confirm this. Nevertheless, the increased root to shoot ratio in presence of *P. fluorescens*, independently on *G. intraradices*, but conditioned by plant line and P amendment, seems to be in conformity with the expected for an increase in auxin (Hermans *et al.* 2006; Hodge *et al.* 2009). If this is a result of bacteria synthesis of auxin or other factor stimulated by *P. fluorescens* is not possible to conclude and require further test. Additionally, the strain used in the present study has been previously reported to enhance plant growth by antagonism against root pathogenic fungi (Kristek *et al.* 2008). It is possible that when *P. fluorescens* itself is not deleterious to the root (Alström 1987; Bolton & Elliott 1989; Watt *et al.* 2003), its colonization along the root can become protective against root pathogenic fungi and indirectly enhance plant growth (Lugtenberg & Kamilova 2009). Although the substrate was initially sterilized, most pathogens cannot be excluded from the greenhouse environment: airborne spores can enter through doors and screens, soilborne pathogens enter through dust, zoosporic pathogens enter through irrigation water, and insects carry fungal inoculum or transmit viruses (Paulitz & Belanger 2001). The greenhouse where the experiment was set up had S1 security and is generally subject to weekly pest control by pesticide application. However, the area reserved to the present experiment was especially maintained without any pesticide application to avoid deleterious effects on the inoculated soil microorganisms, and is likely that pathogens occurred because the greenhouse environment was not aseptic.

The plant dry mass of the *35S:CKX2* on low P was not affected by co-inoculation with *G. intraradices* and *P. fluorescens* compared with the non-inoculated control. The total root length, surface area and volume showed a slight trend to decrease, while the average root diameter was not affected. The specific root length was decreased compared with the non-inoculated control, but enhanced when

comparing with the microbial single inoculations. The root to shoot ratio was drastically enhanced compared with the control, mostly due to shoot reduction, and the N content in the shoot was not affected by any changes in root morphological parameters or root to shoot ratio. As occurred in the root morphology in the wild type on low P, it occurred a compensatory mechanism in the co-inoculation which allowed the plant to sustain the same N content in the shoot as well as the whole plant dry mass compared with the non-inoculated control, but in the *35S:CKX2* on low P the most notable mechanism was the enhancement of the root to shoot ratio, rather than enhanced root morphological parameters. The mycorrhizal root colonization was also enhanced almost threefold (28%) in the co-inoculation, compared with *G. intraradices* alone (10%). The enhanced mycorrhization was not accompanied by increased N content in the shoot, supporting the hypothesis that *G. intraradices* colonization did not had an important role in plant N uptake. The plant-fungus relation is still a commensalism, as referred above and described by Johnson *et al.* (2010). When the plant is CK deficient, the microbial co-inoculation tended to slightly suppress the root morphological parameters, contrastingly to the observation in the wild type. This suggests that CK played a role in the microbial-plant signaling on the enhancement of root morphology. The balance between auxin and CK play an important role in the root growth and morphology (Hodge *et al.* 2009). AMF and *P. fluorescens* have been suggested to directly enhance CK in the plant (de Salamone *et al.* 2001; Shaul-Keinan *et al.* 2002), and a reduced CK plant mutant, due to CK oxidase activity, is likely more sensible to auxin inductions but more resistant to CK. In the co-inoculation, the mycorrhization increased in presence of *P. fluorescens*, and although *P. fluorescens* alone enhanced root morphology, when its activity result also in increased mycorrhization, is likely that there was a counteracting mechanism enhancing lateral/fine roots possibly via local auxin stimulation (Hause *et al.* 2007; Gutjahr *et al.* 2008), and increasing the specific root length in comparison with the single inoculations.

35S:CKX2 on high P

The plant dry mass of *35S:CKX2* growing on high P was reduced by the microbial inoculations, alone or in combination. *G. intraradices* alone reduced the total root length, surface area and volume, and tended to reduce the average root diameter. Despite the drastic root to shoot ratio increase, the N content in the shoot was reduced, in what it seem a consequence of the reduced root morphological parameters by *G. intraradices*. Here, the drastic increase in root to shoot was not sufficient to compensate the reduced N uptake due to reduced root morphology. *P. fluorescens* reduced the plant dry mass, but did not affect the root morphological parameters; it only tended to enhanced the average

root diameter, but with no consequences on the total root surface area and volume. *P. fluorescens* clearly reduces the N content in the shoot, suggesting that competition for N occurred between the plant and *P. fluorescens*. The co-inoculation had also negative effects on plant dry mass, without major changes in the root morphological parameters, but clearly reduced the N content in the shoot. In the *35S:CKX2* on high P the N limitation seems to explain better the plant growth than the root morphology. If P is not so limited for plant growth and N becomes comparatively limited, the CK deficient plant interaction with the soil microbes was not subject to root morphological changes, possibly due to CK deficient signaling, not allowing root adaption to soil adverse condition and enhance of N uptake. These results reject partially hypothesis 1,1. Although the effects of soil microorganism on plant growth were strongly related with their effect on root morphology, this was not always verified, and other factors such as plant nutrient limitation induced by other interactions with soil microbes are equally important. This conclusion is not surprising, several studies have suggested that soil microbes can affect plant growth by interfering with plant nutritional status (Kaye & Hart 1997; Smith & Read 2008; Lugtenberg & Kamilova 2009). Nevertheless, the results confirm hypothesis 1.2. The effects of soil microorganism on root morphology depended on P amendment and on the tobacco endogenous CK content. Additionally, it was also dependent on the interaction between the soil microorganisms.

Mycorrhization

The tobacco *35S:CKX2* was more susceptible to root colonization by *G. intraradices* than the wild type, confirming the hypothesis 2.1. In general, P deficient soils are favorable to AMF colonization (Smith & Read 2008). P starvation induces plants to activate several mechanisms to overcome this nutrient limitation, which is generally achieved by developing a more profusely diffused root system, with enhanced root hair formation, increased root exudation and/or enhanced mycorrhization (Hodge *et al.* 2009; Richardson *et al.* 2009). CK is involved in the signaling between root and shoot when the plant is P deficient (Franco-Zorrilla *et al.* 2005), and seems to have an important role in the activation of response mechanisms to P starvation stress (Hermans *et al.* 2006). AM plants accumulate more CK than non-mycorrhizal plants in both shoots and roots. However, the increased levels occur only in a very late phase of mycorrhization (Hause *et al.* 2007). It has been assumed that the increase in CK levels could be due to increased phosphate nutrition in AM plants resulting in CK production, presumably synthesized in the additional root primordial. Shaul-Keinan *et al.* (2002) reported that following AM fungal colonization, specific cytokinin compounds were being synthesized irrespective of the improvement of P level associated with AM symbiosis. Adding to the current literature that emphasize the role of CK in the AM symbiosis, the present study demonstrates that endogenous CK shortages can have a positive effect on *G. intraradices* colonization in tobacco roots. Additionally, the *35S:CKX2*-expressing plants are known to induce the faster-growing roots due to their CK-deficiency (Werner *et al.* 2001), but their roots have also a strongly reduced content of soluble sugars compared with the wild type (Werner *et al.* 2008). Plants supply C to the fungi via soluble sugars mainly as hexoses (Johnson 2010). This suggests that the mechanism enhancing AMF colonization in the *35S:CKX2* is not associated with surplus of soluble sugars but rather by other mechanism involving CK signaling.

P. fluorescens enhanced colonization of tobacco root by *G. intraradices*, independently of plant line or P amendment, suggesting that this bacteria strain is a mycorrhiza helper bacterium (MHB). Several MHB have been described for AMF but no examples refer to a rhizobacteria isolated from a non-mycorrhizosphere (Frey-Klett *et al.* 2007). Generally, MHB can promote the establishment of symbiosis by stimulating mycelial extension; increasing root-fungus contacts and colonization; and reducing the impact of adverse environmental conditions on the mycelium of the mycorrhizal fungi (Frey-Klett *et al.* 2007). The mechanisms by which bacteria stimulate AM colonization are still poorly understood, but there are multi mechanism involved, including direct effect on the fungal growth (Frey-Klett *et al.* 2007), direct effect on the plant host by increasing root cell permeability and hormone synthesis, or indirectly

by decreasing the activity of soil born competitors. Given the origin of the bacteria strain used in the preset study, AMF was not present in its original ecological context, and direct stimuli are less probable than the indirect. For example, this strain is a successful antagonist against pathogenic fungi, e.g. *Pythium ultimum*, that are known to compete with *G. intraradices* (Starnaud *et al.* 1994; Kristek *et al.* 2008; Wehner *et al.* 2010). Therefore, it is plausible that the antagonistic activity against soil born competitors indirectly stimulated AMF proliferation.

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